การสืบหายีนต้านมะเร็งในมะเร็งโพรงหลังจมูก

นางสาว ปฐมวดี ญาณทัศนีย์จิต

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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TO SEARCH FOR NEW CANDIDATE TUMOR SUPRESSOR GENES IN NASOPHARYNGEAL CARCINOMA (NPC)

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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Thesis Title	TO SEARCH FOR NEW CANDIDATE TUMOR SUPPRESSOR GENE NASOPHARYNGEAL CARCINOMA (NPC)
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Field of Study	Biomedical Science
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ปฐมวดี ญาณทัศนีย์จิต : การสืบหายีนต้านมะเร็งในมะเร็งโพรงหลังจมูก. (TO SEARCH FOR NEW CANDIDATE TUMOR SUPPRESSOR GENE IN NASOPHARYNGEAL CARCINOMA) อ. ที่ปรึกษา: ผศ. ดร. วิโรจน์ ศรีอุฬารพงศ์, 76 หน้า.

ในการสืบหายีนต้านมะเร็งในมะเร็งโพรงหลังจมูกศึกษาจากการสืบค้นข้อมูลการแสดงออกของยีนเปรียบเทียบระหว่างเซลล์มะเร็งโพรงหลังจมูกกับเซลล์ปกติโดยเลือกยีนที่มีการแสดงออกของยีนในเซลล์มะเร็งต่ำกว่าในเซลล์ปกติ โดยในการศึกษาครั้งนี้สามารถเลือกยีนที่น่าสนใจได้ทั้งสิ้น 8 ยีน และแบ่งออกเป็น 2 กลุ่ม โดยในกลุ่มที่หนึ่งประกอบด้วย *RARRES1, HRASLS3, LOH11CR2A* และ *MCC* ซึ่งเป็นยีนต้านมะเร็งในมะเร็งชนิดอื่น และยีนในกลุ่มที่สองประกอบด้วย *CCNA1,CLMN, EML1* และ *TSC22* ซึ่งเป็นยีนที่อยู่ในบริเวณที่มีการขาดหายไปของโครโมโซมซึ่งเป็นสาเหตุใหญ่ของการกดการทำงานของยีนต้านมะเร็ง เนื่องจากยีนทั้ง 8 มีการแสดงออกของยีนในมะเร็งโพรงหลังจมูกต่ำกว่าเซลล์ปกติ ซึ่งสาเหตุหลักอีกสาเหตุหนึ่งมาจากการเติมหมู่เมทิลที่บริเวณโปรโมเตอร์ของยีนต้านมะเร็ง ดังนั้นในการศึกษาครั้งนี้จึงทำการสืบหายีนต้านมะเร็งยีนใหม่ในมะเร็งโพรงหลังจมูกโดยการตรวจสอบการเติมหมู่เมทิลบริเวณโปรโมเตอร์ของทั้ง 8 ยีน โดยเปรียบในเซลล์มะเร็ง กับ เซลล์เม็ดเลือดของคนปกติ ซึ่งผลการคัดกรองพบว่ามีเพียง 3 ยีนคือ *CCNA1, RARRE1* และ *HRASLS3* เท่านั้นที่พบการเติมหมู่เมทิลในเซลล์มะเร็งแต่ไม่พบในเซลล์เม็ดเลือดขาวของคนปกติ จากนั้นจึงทำการตรวจสอบการเติมหมู่เมทิลของทั้ง 3 ยีน โดยเปรียบเทียบในเซลล์มะเร็งประมาณ 100 ราย เซลล์เม็ดเลือดขาวของคนปกติ 30 ราย และ เซลล์ปกติ 20 ราย พบว่า มีการเติมหมู่เมทิลที่บริเวณโปรโมเตอร์ของ *CCNA1* 57%, *RARRES1* 54% และ *HRASLS3* 24% นอกจากนี้ยังพบว่าใน primary cell culture มีการเติมหมู่เมทิลของ *CCNA1* 100%, *RARRES1* 83% และ *HRASLS3* 17% จากการศึกษาในครั้งนี้ทำให้สามารถสรุปได้ว่า *CCNA1, RARRES1* และ *HRASLS3* เป็นยีนต้านมะเร็งยีนใหม่ในมะเร็งโพรงหลังจมูก

สาขาวิชา ชีววิทยาศาสตร์ ลายมือชื่อนิสิต ปฐมวดี ญาณทัศนีย์จิต
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KEY WORD: PROMOTER HYPERMETHYLATION/ TUMOR SUPPRESSOR GENE / NASOPHARYNGEAL CARCINOMA

PATTAMAWADEE YANATATSANEEJIT: TO SEARCH FOR NEW CANDIDATE TUMOR SUPPRSEEOR GENES IN NASOPHARYNGEAL CARCINOMA.

THESIS ADVISOR: ASSIST PROF VIROTE SRIURANPONG, M.D. Ph.D., 76 pp.

To search for new candidate tumor suppressor gene in nasopharyngeal carcinoma (NPC), we studied from the expression profiling compare between tumor and normal tissues. We selected 8 down regulated genes which can be divided into 2 groups, the first group is the genes which are tumor suppressor genes in other cancer; *RARRES1' HRASLS3, LOH11CR2A* and *MCC*. The other is the genes which are in the critical region of loss of heterozygosity(LOH); *CCNA1, CLMN, EML1* and *TSC22*. Promoter hypermethylation, which is the major factor, leads to suppress gene expression. In this study, we detected promoter hypermethylation on promoter of the 8 candidate genes in NPC, normal leukocytes. Only 3 genes had promoter hypermethylation in NPC but not in normal leukocyte; *CCNA1, RARRES1* and *HRASLS3*. We next increased the sample of NPC to around 100 cases, normal leukocyte 30 cases and normal epithelium cells 20 cases, there was promoter hypermethylation on *CCNA1* 57%, *RARRES1* 54% and *HRASLS3* 24%. Moreover, we could detect promoter hypermethylation *onCCNA1* 100%, *RARRES1* 83% and HRASLS3 17% in primary culture cells. This study, we concluded that *CCNA1, RARRES1* and *HRASLS3* might be act as new tumor suppressor genes in NPC.

Field of study Biomedical Science Student's signature Pattamawadee Yanatatsaneejit

Academic year 2007 Advisor's signature....Virote Sriuranpong....

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LIST OF ABBREVIATION

NPC	=	Nasopharyngeal Carcinoma
EBV	=	Epstein Barr Virus
MSP	=	Methylated specific PCR
COBRA	=	Combined bisulfited restriction analysis
CCNA1	=	Cyclin A1
RARRES1	=	Retinoic acid receptor responder 1
HRASLS3	=	H-ras like suppressor 3
PCR	=	Polymerase Chain Reaction
LOH	=	Loss of heterozygosity
DNMT	=	DNA methyltransferase
BRCA1	=	Breast cancer 1
BRCA2	=	Breast cancer 2
pRB	=	Retinoblastoma protein
WT1	=	Wilms'tumor suppressor gene
APC	=	Adenomatous polyposis coli
NF1	=	Neurofibromin 1

NF2	=	Neurofibromin 2
P53	=	Protein 53
IRF1	=	Interferon Regulatory Factor
PCNA	=	Proliferating Cell Nuclear Antigen
BCL-2	=	Integral membrane protein
TS	=	Tumor suppressor
UTR	=	Untranslated region
WBC	=	White Blood Cell

CHAPTER I

INTRODUCTION

Background and Rationale

Nasopharyngeal carcinoma (NPC) is one of the most common cancers in Asia, especially in Southern China and Southeast Asia. The prevalence in Thailand is 3-10/100,000. (1) Radiotherapy is an effective treatment for NPC, and more than 80% of patients with early disease are curable. Unfortunately, most of the NPC patients are diagn sed at later stages, where treatment is much less effective and more difficult. If these patients can be diagnosed earlier or if relapses can be predicted sooner, clinical management would be improved greatly. (2)

Previous etiological studies demonstrated that the development of NPC might be attributable to a complex interaction of EBV infection, dietary exposure to chemical carcinogens and genetic factors. (2-4) Relatively little information of the NPC associated genetic alteration is known. Hence, identification of genetic changes in this cancer is crucial in revealing the molecular basis of NPC tumorigenesis. Previous studies have shown that loss of function of tumor suppressor genes is one of the causes of NPC. In general, loss of function of tumor suppressor genes results from loss of function of two alleles, one is caused by loss of heterozygosity (LOH), and the other is promoter hypermethylaton or mutation. (5-8) Many studies revealed promoter hypermethylation in several cancers including NPC. (9-11) In NPC, methylation of the tumor suppressor genes such as *RASSF1A*, *TSLC1* and *HIN-1* has been found in primary tumors and correlated to gene silencing. (12-15)

In our previous work, we used Laser capture microdissection (LCM) to procure cells from tumors or adjacent nonneoplastic tissues from NPC for transcriptional profiling by high density cDNA microarray. This comparative analysis between normal nasopharyngeal epitheliums and tumor tissues revealed numerous differentially expressed genes. The number of genes differently expressed between them was 477 genes whose expression level was up-or down-regulated in cancer type. (16) In this

study, we selected 8 down regulated genes from microarray data to search for new candidate tumor suppressor genes. Eight genes were divided into 2 criteria, the first group was genes located on critical region of LOH on chromosome 13 and 14; *CCNA1*, *TSC22*, *EML1* and *CLMN*. (17) The other group was genes which are tumor suppressor genes in other types of cancer; *HRASLS3* (in lung cancer) (16,18), *RARRES1* (in lung cancer, prostate cancer and head and neck cancer) (16,19-21), *LOH11CR2A* (in lung cancer and breast cancer) (16,22) and *MCC* (in colon cancer). (16) To verify whether methylation of these 8 genes is specific to tumor cells, all of them are detected through analysis of promoter methylation in tumor cells and normal white blood cells. We found that *CCNA1*, *HRASLS3* and *RARRES1* are specific to tumor cells. Taken together, these three genes may be new candidate tumor suppressor genes.

Research questions

1. From our microarray data, which down-regulated genes may be putative tumor suppressor genes?
2. Do promoter hypermethylation of these candidate tumor suppressor genes correlate to clinical data?

Objectives

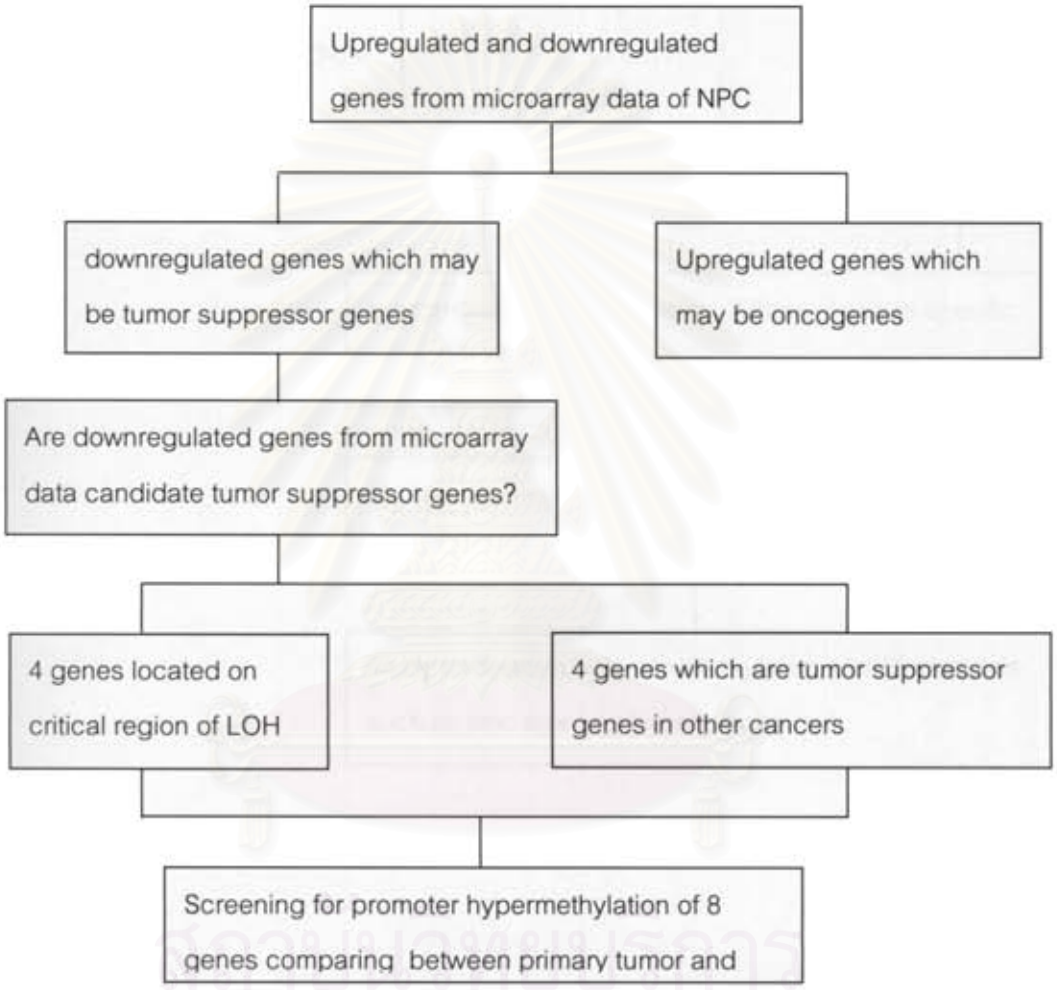
1. To search for new candidate tumor suppressor genes in NPC.
2. To study the correlation between promoter hypermethylation on putative tumor suppressor genes and clinical data.

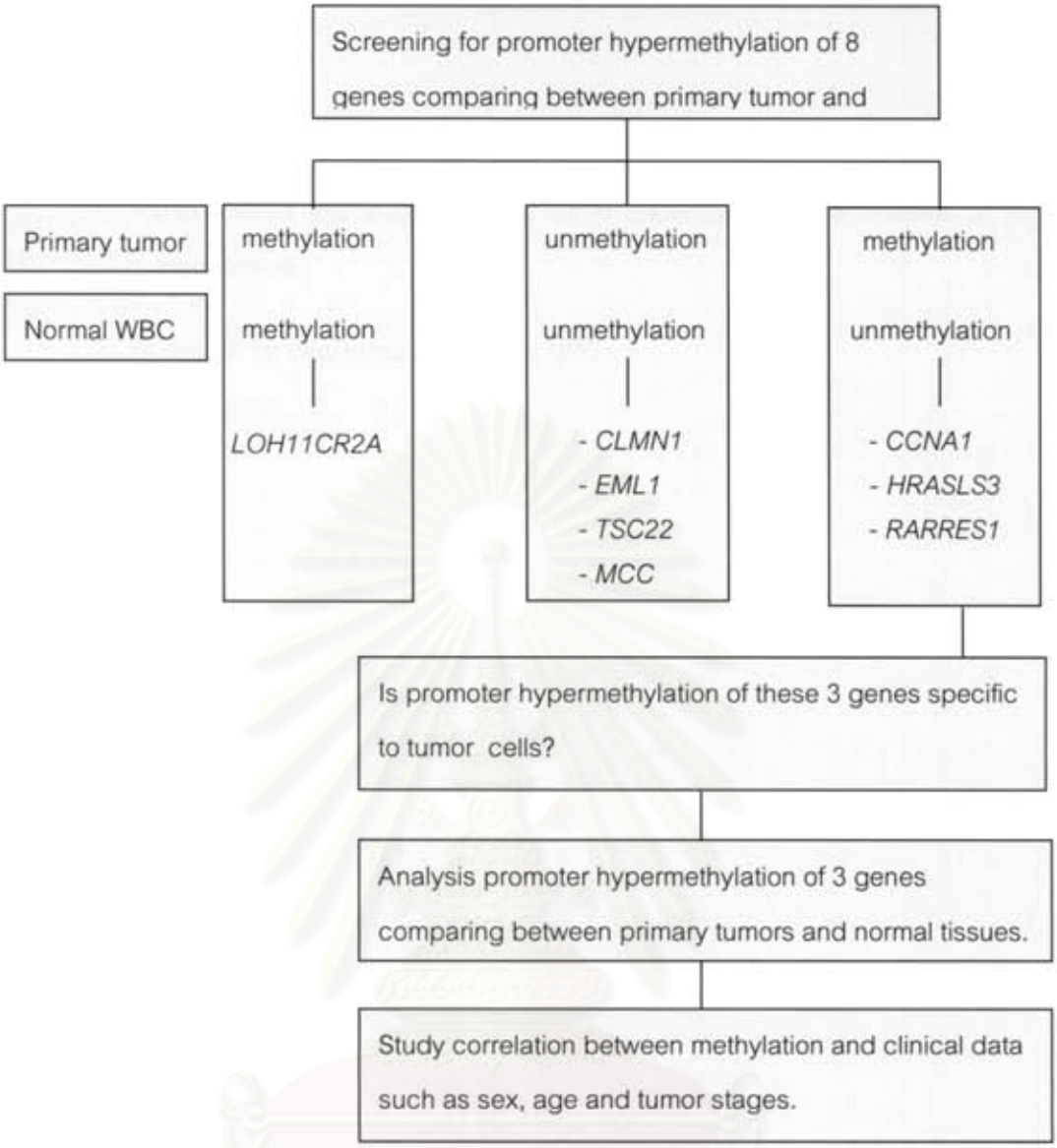
Hypotheses

1. From microarray data, some down regulated genes are tumor suppressor genes.
2. If there is promoter hypermethylation on down-regulated genes in NPC cells not in normal cells, these genes may be putative tumor suppressor genes.

3. There may be the correlation between promoter hypermethylation of putative tumor suppressor genes and clinical data such as sex, age and tumor stage.

Conceptual framework





Expected Benefit and Application

1. The results of this study will help us to understand the mechanism of NPC tumorigenesis.

2. To search for new tumor markers in NPC

CHAPTER II

REVIEW OF RELATED LITERATURE

Nasopharyngeal Carcinoma (NPC)

Histopathology

NPC is distinguished from other head and neck cancers by its histopathology, epidemiology, clinical characteristics, and therapy. The World Health Organization (WHO) classification subdivides NPC into three variants according to the predominant histological pattern. WHO type I is a keratinising squamous cell carcinoma similar to carcinomas that arise from other sites of the head and neck. This type is not common in endemic areas. WHO type II is a nonkeratinising squamous cell carcinoma while WHO type III is an undifferentiated carcinoma. Both types are referred to as lymphoepithelioma or Schminke tumors, squamous epithelial tumors with heavy lymphocyte infiltration, respectively. They are common in endemic areas. (23)

Epidemiology and Evidence of Susceptibility

NPC is a rare disease in most parts of the world. It occurs with a high incidence in Southern China, Southern Asia, and other high-risk populations including North Africa and Eskimos. The aetiological factors of endemic NPC include EBV infection, genetic susceptibility, and environmental factors. (24, 25) EBV association in NPC refers to those situations in which the viral genome or gene products are detected within tumor cells. This virus exists within tumor cells in a monoclonal form, with every viral episome carrying an identical number of terminal repeat regions. This suggests that such episome was derived from a single infected cell. EBV infection can cause a variety of diseases as a consequence of primary and latent infection. The virus primarily infects the oropharyngeal epithelium. EBV then replicates, lyses, and infects B-lymphocytes. After infection, the virus enters the lytic phase and causes diseases ranging from mild self-limited illness in children to infectious mononucleosis (IM) in adolescences and adults. However, in some cells the virus will switch to the latent phase. These latently

infected B-lymphocytes have the inclination to turn into lymphoma such as Burkitt's lymphoma (BL), Hodgkin's disease (HD) or can play an important role in the dissemination of infection in the nasopharynx. Consequently, this virus can induce NPC development (Figure1). (26-28) In addition to EBV, environmental factors include the ingestion of Chinese style salted fish, especially during childhood. Several carcinogenic volatile nitrosamines have been detected in Chinese salted fish. Besides salted fish, exposures to other preserved food products, such as salted shrimp paste, fermented soybean paste and various types of salted vegetable, have been shown to be related to NPC risk in Chinese. (24,29,30) Among non-dietary environmental exposures, tobacco smoking has been suggested to be a risk factor for NPC. (31-35)

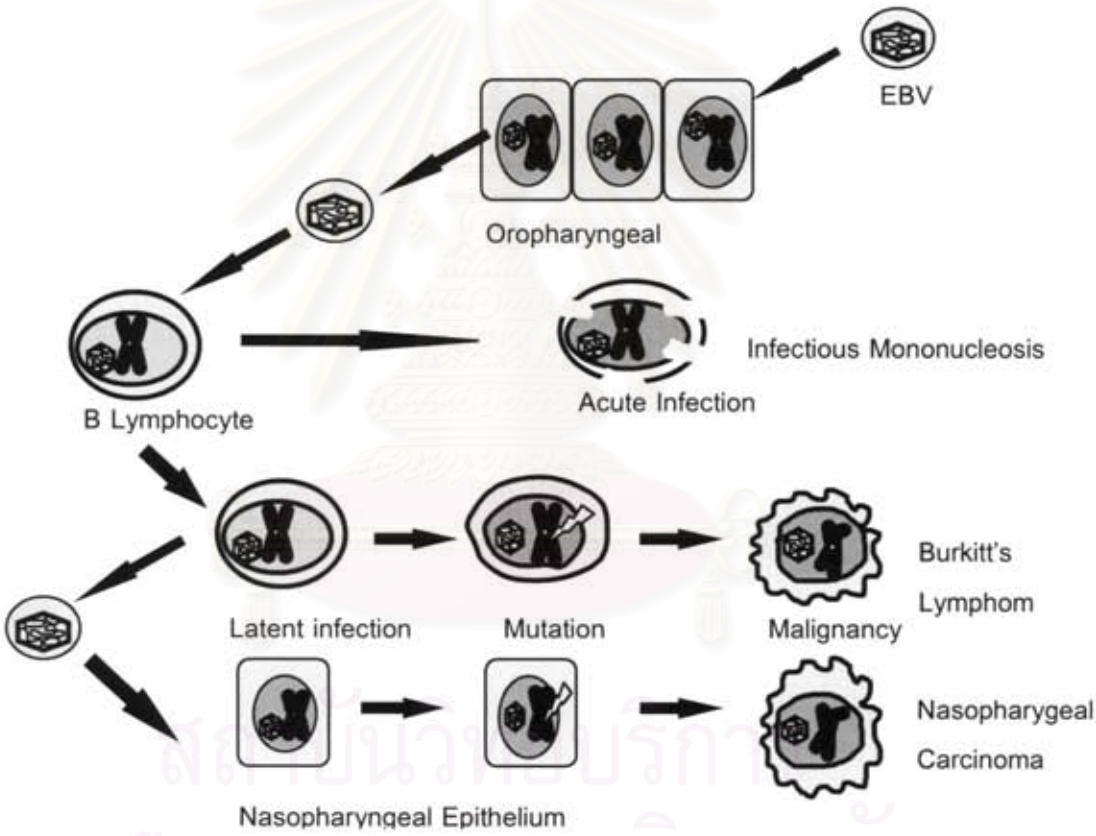


Figure1 A model of the Epstein-Barr Virus infection and disease development

Mechanism of Nasopharyngeal Carcinogenesis

NPC involves multiple genetic alterations. The genetic alterations include the inactivation of tumor suppressor genes and activation of oncogenes. For example, previous studies reported frequent allelic loss on chromosome 3, 9, 11, 13 and 14, suggesting potential locations for essential tumor suppressor genes. (36) In addition to mutations, viral carcinogenesis involves interactions between viral oncoproteins and its host. Latent membrane protein 1 (LMP1) is a well-known viral oncoprotein from EBV. It can inhibit p53-mediated apoptosis through the induction of *A20* anti-apoptosis gene. LMP1 expression in epithelial cells activates transcription factor NF-κB. (37) Therefore, the upregulation of *A20* expression is a result of NF-κB activation. Interestingly, LMP1 has been shown to engage signaling proteins for the tumor necrosis receptor (TNFR). Activation of this signaling molecule by LMP1 could mimic ligand binding to this family of receptors, and therefore subsequent induction of A20 may be important for the inhibition of apoptosis induced by activation of this pathway. (38) Additionally, LMP1 also induces expression of EGFR (epidermal growth factor receptor), leading to a deregulation of cellular growth control. The effect of LMP1 resembles those activated Ras molecules, suggesting a common biochemical pathway for these oncoproteins. Since overexpression of the EGFR by LMP1 could mimic an activated Ras pathway, LMP1 may exert its transforming effects in epithelial cells via deregulation of the EGFR. (39) In conclusion, LMP1 is a cause of increased proliferating signals due to enhanced EGFR expression, as well as decrease cell death due to LMP1 induced A20 expression can contribute to development of epithelial malignancies.

Cancer genes

Cancer genes can be divided into two broad categories. The first group is oncogenes, whose normal activity promotes cell proliferation. Gain of function mutations of these genes in tumor cells create forms that are excessively or inappropriately active. A single mutant allele may affect the phenotype of the cell. The nonmutant versions are properly called proto-oncogenes. The other group is tumor suppressor (TS) genes. TS gene products inhibit events leading toward cancer. Mutant versions in cancer cells

have lost their function. Some TS gene products prevent inappropriate cell cycle progression, some steer deviant cells into apoptosis, while others keep the genome stable and mutation rates low by ensuring accurate replication, repair and segregation of the cell's DNA. Both alleles of TS gene must be inactivated to change the behavior of the cell.

Tumor suppressor genes can be divided into 2 classes.

The Class I Tumor Suppressor Genes

Since the identification of the first tumor suppressor gene,more than 20 other genes have been shown to be mutated or deleted in tumours, Retinoblastoma tumor suppressor gene (*pRB*), Protein 53 (*p53*), Wilms'tumor suppressor gene (*WT1*) Breast cancer1(*BRCA1*), Breast cancer2 (*BRCA2*) Adenomatous polyposis coli (*APC*), Neurofibromin1(*NF1*), and Neurofibromin2 (*NF2*). (40, 41) The genes disrupted in a majority of human cancers are *RB1*, and the *TP53* gene. *BRCA1* and *BRCA2* were demonstrated to play an important role in the heredity of ovarian and breast cancers. (42).

The *RB1* gene was the first tumor suppressor gene to be isolated and cloned. (43)The product of this gene, the retinoblastoma protein, pRB, is a nuclear phosphoprotein which mediates progression through the first phase of the cell cycle, playing a major role in the control of cell division and differentiation. (44) Cytogenetic studies of chromosomal alterations in a childhood retinoblastoma, and in breast, lung and pancreatic cancers demonstrated a correlation between tumorigenesis and chromosomal aberrations on chromosome 13q14 where the *RB1* gene is located.(45)The inactivation of one of the *RB1* alleles by point mutation or deletion was demonstrated to be often accompanied by loss of heterozygosity (LOH) on chromosome 13. (46) In order to explain the nature of retinoblastoma formation, Knudson suggested the so-called "two-hit hypothesis". He proposed that two inactivating mutations affecting both copies of a gene are necessary for retinoblastoma development. The first could be either a germline or somatic mutation, whereas the second mutation is always somatic. This hypothesis illustrated how somatic and inherited mutations might collaborate in tumorigenesis, and also proposed that mutations of tumor suppressor genes have a recessive character, behave recursively at the cellular level.

The *TP53* tumor suppressor gene was demonstrated to carry homozygotic somatic alterations in roughly 50% of all human tumours. Mutations in the single copy *TP53* are the most frequent genetic changes yet shown in human cancers and occur in 70% of all tumors. Further investigations revealed that in contrast to the pRB protein and Knudsons "two-hit hypothesis", the *TP53* carries mutations leading to development of cancer in a dominant negative fashion. (47)

The *TP53* encodes a transcription factor activated in response to physical or chemical stress. The p53 protein controls induction of apoptosis, cell cycle progression into G1 and G2 phases, modulation of DNA replication and repair, preventing proliferation of cells with damaged genetic material. Overexpression of wild type TP53 in different cell types leads to growth inhibition (48), or to the induction of apoptosis in squamous carcinoma cell lines (49). The major down-stream p53 effector participating in the control of the cell cycle check-points is the cyclin dependent kinases inhibitor $p21^{WAF1}$, functioning as a tumour suppressor itself (50; 51). Other p53 effectors playing a critical role in apoptosis signalling are the death signalling receptor Apo-1/Fas (52), the repressor of apoptosis Bcl-2, its inhibitor BAX-1 (51), and the death receptor DR5. Several members of the DNA repair machinery, for example, auxiliary subunit of polymerase ^{TM} (PCNA), and replication protein A (RPA) were also described as p53 targets.

The Class II Tumor Suppressor Genes

The class II of tumor suppressors is represented by genes which, unlike class I, are not mutated during tumorigenesis but rather have sustained a blockage of their expression through diverse mechanisms (53). Interestingly, that some genes exhibit features of class II tumor suppressors in one type of cancer, whereas in other type they are known to belong to the class I tumor suppressors. Thus, allelic loss of *IRF1* occurs frequently in the acute myeloid leukemia, myelodysplastic syndrome (54), and gastric cancer (55), whereas Interferon Regulatory factor 1 (*IRF1)* is described as a class II tumor suppressor in ovarian cancer. (56) Dysfunction of maspin via mutation was identified in prostate cancer (57), whereby down-regulation of this gene is characteristic for many other tumors where the gene is not mutated. (58) An important feature of the

class II tumor suppressor genes is that their down-regulation is reversible. The normal genes are present, and their re-expression might be induced by drugs or other treatments. This finding makes such genes attractive targets for cancer therapy. It is known that inactivation of the expression takes place on the transcriptional and translational levels during cancer progression. However, up until now the mechanisms of the gene silencing have not yet been elucidated in much detail.

Loss of function of tumor suppressor genes

The background to our understanding of TS genes is described by Knudson's work in 1971 on retinoblastoma; Knudson's two-hit hypothesis. This hypothesis explained the mechanism, which inactivates TS genes, leading to cancer as described in figure 2

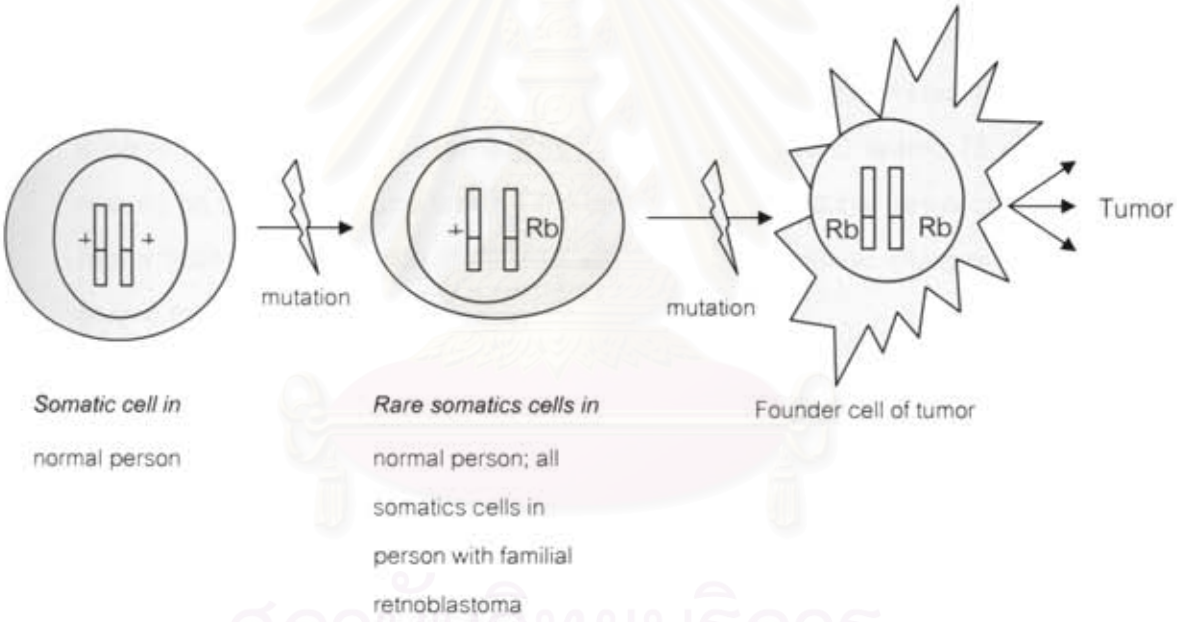


Figure 2 : Knudson's two-hit hypothesis

Knudson's study has shown that TS genes are recessive genes. Therefore, loss of function of these genes are resulted from inactivation of both alleles. Loss of function of these genes usually results from loss of heterozygosity (LOH) along with promoter

hypermethylation. LOH along with mutation is rare. Sometimes loss of function results from homozygous deletion.

There are many groups study the cause of loss of function of TS. The first group revealed the methylation status of the promoter region of multiple TS gene at chromosome 3p in esophagus squamous cell carcinoma. (5) The second group revealed the concordance between LOH and methylation was 56% for breast cancer cell lines and 48% for primary breast carcinoma, respectively. For lung cancer, the concordance between LOH and methylation was 63% for lung cancer cell lines and 43% for primary NSCLC samples, respectively. (6) The third group studied the LOH of 3p in the neoplastic and nonneoplastic lesion of the nasopharynx from the high risk population (in Southern China from Hong Kong) and low risk population (in Anhui/ Beijing and Toronto). They found that dysplasia from high risk group and normal nasopharyngeal tissues from noncancer individuals in Hong Kong showed 3p LOH on one or more loci examined. This study has suggested that deletion of chromosome 3p is a genetic event in the tumorigenesis of this cancer, and NPC related TS genes are residing on this chromosome arm. (7) The other group showed that loss of chromosome region 9p21 and inactivation of the *P16* are the common genetic changes and occur early in the progression of head and neck cancer. (8)

1. Epigenetics and DNA methylation

Epigenetics

Epigenetics can be defined as the study of genome function that is contained outside of DNA itself and by means of which stable alterations in gene expression are set. Epigenetics is a well-established phenomenon that plays a major role in a diversity of biological processes such as embryonic development, cancer biology, and immune system response among many others. The two most widely studied epigenetic changes are DNA methylation and histone deacetylation. However, others have complicated the situation such as the RNA interference phenomenon, which has proven to be implicated

in transcriptional silencing through small duplex molecules that recruit silencing complexes to the chromatin.

DNA methylation

DNA methylation, the addition of a methyl group to the 5′carbon of deoxycytosine, is one such epigenetic modification found in DNA. (61) It is heritable and involved in regulation of gene transcription. In mammals, the major target for DNA methylation is found in CpG – dinucleotide. CpG dinucleotide is the cytosine located next to a guanine(5′-CpG-3′) . (62) These targets of methylation are not equally distributed in the genome, but found in long CG-rich sequences present in satellite repeat sequences, middle repetitive rDNA sequences, centromeric repeat sequences and CpG islands. CpG islands are sequences longer than 200 bp with a GC content of over 50% (in contrast to a genome-wide average of about 40%) with an observed over expected ratio of CpG being 0.6 or greater. (63) Interestingly, CpG islands are found mainly in the 5′ -regions of house-keeping genes as well as some other tissue -specific expressed genes. They usually extend from the promoter region into the first exon and sometimes into intron 1. (64) Most CpG islands are unmethylated in normal cells; however, there are certain conditions where these sequences become methylated and form a part of gene regulation process. (65) The majority of CpG islands on the inactive X-chromosome in a female cell are methylated, (66) Certain CpG island-like sequences in the vicinity of imprinted genes have also been found to be methylated in an allele-specific manner. (67) Furthermore, it has been found that some CpG islands become methylated with age. (68) While CpG islands are usually unmethylated, other GC-rich sequences, e.g. the centromeric repeat sequences and satellite sequences, are highly methylated in normal cells.

Mechanisms of DNA methylation

DNA methylation is mediated by a family of DNA methyltransferases (Mtases) that include Dnmt1, Dnmt3a, and Dnmt3b. Dnmt1 is a maintenance Mtase that primarily replicates methylation patterns, while Dnmt3a and Dnmt3b are capable of methylating previously unmethylated DNA, referred to as *de novo* methylation. (69) DNA methylation patterns are established during differentiation, and serve to suppress genes unnecessary for the function of the mature cell. This involves *de novo* methylation of DNA, and requires Dnmt3a and Dnmt3b. (70) Their role in mature cells is less clear, but Dnmt3b appears to be necessary for maintaining methylation of pericentromeric heterochromatin . (71) Following differentiation, the patterns are replicated during mitosis by the maintenance DNA Mtase Dnmt1. (69) During mitosis, Dnmt1 recognizes hemimethylated CG dinucleotides in the parent DNA strand, and catalyzes the transfer of the methyl group from S-adenosylmethionine (SAM) to the cytosine residues in the unmethylated daughter DNA strand, producing symmetrically methylated sites and maintaining methylation patterns (Figure 3). (69)

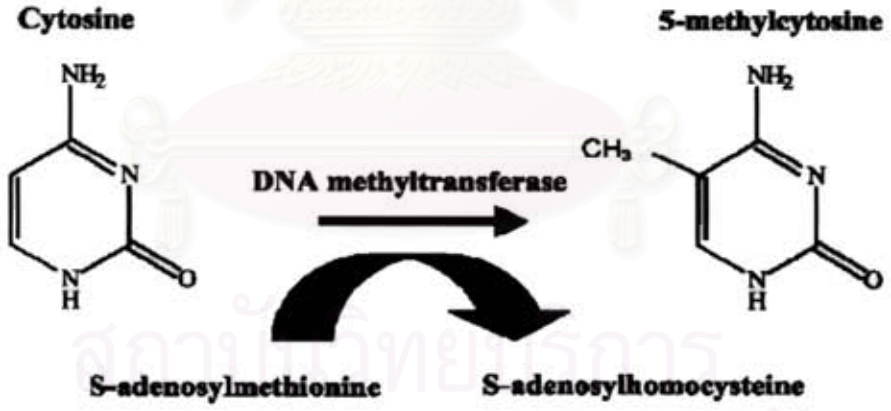


Figure 3: Cytosine methylation. The DNA methyltransferases catalyze the transfer of the methyl group from S-adenosylmethione to cytosine, producing 5-methylcytosine and S- adenosylhomocysteine.

Mechanisms of gene suppression

The methylation of CG sequences can affect nearby gene expression. Hypomethylation of regulatory sequences usually correlates with gene expression, while methylation results in transcriptional suppression. In contrast, methylation of coding sequences generally has little effect on gene expression. (72) Methylation suppresses transcription by at least three mechanisms. Methylation of recognition sequences prevents the binding of some transcription factors such as AP-2. (73) A family of methylcytosine binding proteins has been described, which inhibit binding of transcription factors to promoters (74) as shown Figure 4. Interestingly, all three DNA Mtases can also suppress gene expression directly independent of their methylation activity. (75) Finally, some methylcytosine binding proteins such as MeCP2 and MBD2 can promote chromatin condensation into an inactive configuration through interactions with chromatin inactivation complexes containing histone deacetylases. (74) This can affect gene expression at a distance from the methylated region.

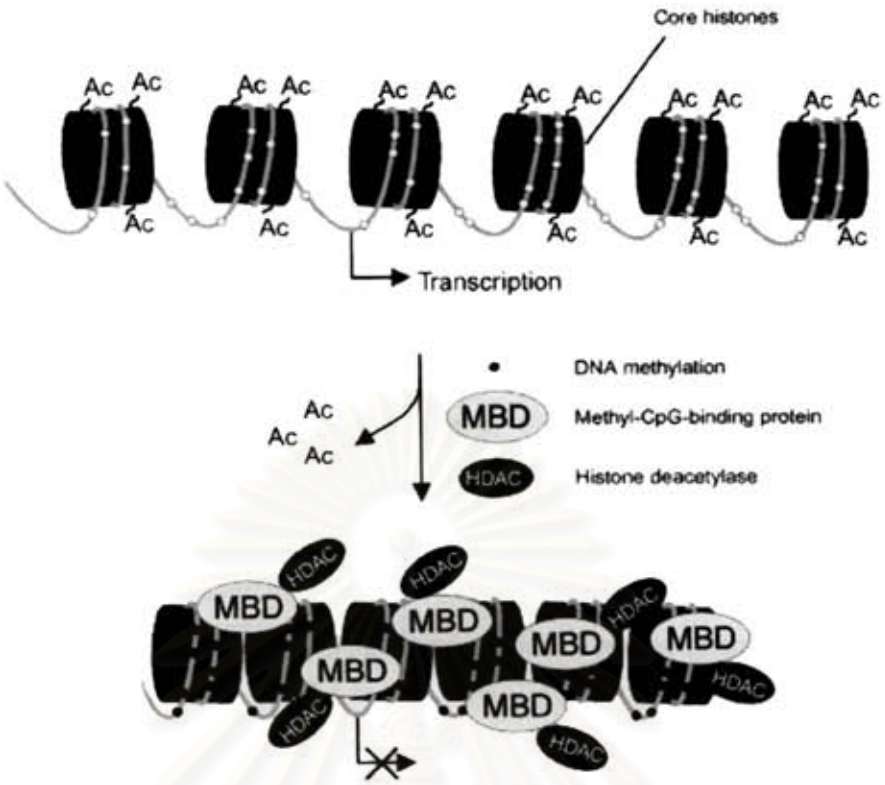


Figure 4 Mechanisms of transcriptional repression by DNA methylation.

Importance of DNA methylation in normal cells

The importance of DNA methylation in the function of normal cells is evidenced by its role in differentiation of development, X chromosome inactivation, genomic imprinting, maintenance of chromatin structure, and suppression of "parasitic" DNA. (75)

DNA methylation in cancer

Tumorigenesis is known to be a multistep process in which defects in various cancer genes accumulate . Virtually every tumor type has revealed an enormous complexity of altered gene functions, including activation of growth-promoting genes as well as silencing of genes with tumor growth-suppressing functions, all contributing to

uncontrolled growth. Cancer gene functions can be classified into six essential alterations in cell physiology, including self- sufficiency in growth signals, insensitivity to growth inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis . It is now clear that the genetic abnormalities found in cancers will not provide the complete picture of genomic alterations. Epigenetic changes, mainly DNA methylation and, more recently, modification of histones, are now recognized as additional mechanisms contributing to the malignant phenotype. The study of these epigenetic changes on a genome-wide scale is referred to as epigenomics (76).

Hypomethylation and hypermethylation are most often used to describe relative states, best understood in relative to an expected, "normal" setting or degree of methylation (i.e. the level of methylation that is seen in nonmalignant, nonaging cells). Hypomethylation and hypermethylation of DNA are relative terms and denote less or more methylation than in some standard DNA. When applied to cancer epigenetics, the standard is normal tissue. However, there are considerable differences in the amounts and distribution of DNA methylation among normal different vertebrate tissues because DNA methylation is not only species specific but also tissue-specific (77).

The discovery of extensive cancer associated DNA hypomethylation in the human genome (78) preceded that of cancer-linked DNA hypermethylation. DNA hypomethylation in cancer often affects more of the genome than does hypermethylation so that net losses of genomic 5-methylcytosine are seen in many human cancers. The biological significance of DNA hypomethylation in cancer is less understood. However, the role in carcinogenesis of cancer linked hypermethylation of transcription control regions is clear because of the consequent transcriptional silencing of genes important for prevention of cancer (tumor suppressor gene).

Many studies have revealed promoter hypermethylation which is an alternative way to inactivate tumor suppressor genes in cancer. The first group determined the frequency of aberrant promoter methylation of the genes retinoic acid receptor β-2 (*RAR–β*), tissue inhibitor of metalloproteinase3 (*TIMP-3*), $P^{16INK4A}$,O^{6}-methyltransferase (*MGMT*), death- associated protein kinase (*DAPK*), E-cadherin (*ECAD*), $P14^{ARF}$, and glutathione S-transferase P1(*GSTP1*) in Non-Small Cell Lung Cancers (NSCLC). (9) The

second group suggested the methylation of *FHIT* is a useful biomarker of biological aggressive disease in patients with NSCLC. (10) The third group suggested that hypermethylation of the *RAR*$-\beta$ and *FHIT* may play an important role in the early stage of esophageal squamous cell carcinogenesis. (5) The other group indicated that silencing of the *RUNX3* gene plays an important role in pathogenesis of lung cancer, and aberrant methylation is an important mechanism of inactivation of the *RUNX3* gene in lung adenocarcinogenesis. (11) In addition, there are several studies showed promoter hypermethylation of tumor suppressor genes in NPC. The first group suggested that *THY1* was a good candidate tumor suppressor gene in NPC. It mapped close to a previously defined11q22-23 NPC critical region. Gene expression and protein analyses showed that *THY1* was not expressed in tumor segregant and NPC cell lines whereas it was exclusively expressed in the non-tumorigenic cells. The mechanism of *THY1*gene inactivation in these cell lines was attributed to hypermethylation. (14) The second group suggested that *CHFR* which is one of the mitotic checkpoint regulators and it delayed chromosome condensation in response to mitotic stress was common event in NPC cells which may be due to hypermethylation of the gene promoter region. (2) The third group found *RASSF1A*, which located on a 120 kb minimal deletion region on 3p21.3 was frequently inactivated by promoter hypermethylation in NPC. (15) The other group showed that silencing of *TSLC1* gene expression in NPC was associated with promoter hypermethylation. *TSLC1* gene functions as a single transmembrane glycoprotein that is involved in cell-cell aggregation through homophilic trans-interaction. It is currently reported to be a tumor-suppressor gene in human non-small cell lung cancer (NSCLC). (12)

Cyclin A1 (*CCNA1*)

The cyclins form a large protein family involved in the regulation of eukaryotic cell cycle. Cyclin binding is a key event required for activation of cyclin-dependent protein kinases (CDKs), which regulate progression between phases of the cell cycle. Several CDKs function at different stages of the cell cycle and the activities of CDKs are regulated by various cofactors and modifying enzymes. The D cyclin-associated CDK4 and CDK 6 are the earliest CDKs, being activated in G1 phase. CDK2 binding to cyclins

E and A is then activated before S phase. CDK1 (also known as CDC2) in association with cyclins A and B function at the G_2/M transition. (79) Human cyclin A forms complexes with both CDK2 and CDK1. The activities of CDK2-cyclin A and CDK1-cyclin A are required for entry into S and M phases, respectively. (80)

Human cyclin A1 is the second cyclin A type. It was firstly isolated and characterized by Rong Yang, Roberta Morsoetti and H. Phillip Koeffler in 1997. Cyclin A1 has 48% identity with human cyclin A and is located at chromosome 13q12.3-q13, approximately 1000 kb from the sequence-tagged site marker *WI*-3374. (81)

Cyclin A1 differs from the cylin A2 (also known as cyclin A), Its expression increases at the entry into S phase of previously synchronized leukemic cells (81). In G_2M, cyclin A1 expression and cyclin A1-CDK2 kinase activity reach their maximum levels. Cyclin A1 is detectable throughout the cell cycle in contrast to cyclin A2. Cyclin A2 is a key regulator of the cell cycle in mammalian cells. It is ubiquitously expressed and essential for progression through the cell cycle. (82) Cyclin A2 involved in both S phase and G_2M transition through its association with CDKs. Cyclin A2 associated with CDK2 at the onset of DNA replication in S phase and with CDK1 mainly at G_2M transition. (80)

Cyclin A1 expression is tissue-specific, and high levels of expression are restricted to testis in the healthy organism in humans (83) to eggs and early embryos in Xenopus (84), and to the germline in mice. (83) Cyclin A1 is expressed shortly before or during the first meiotic division in spermatogenesis, and male cyclin A1 knockout mice are infertile. (86) Spermatogenesis is arrested prior to entry into metaphase I associated with inactive cyclin B- CDK1 complexes and therefore loss of M-phase factor activity. (87) Cyclin A1 expression is also diminished in patients suffering from infertility. (88) It interacts with the cell cycle regulators E2F and pRB in SAOS-2 cells (82), which indicates a tissue specific role in mitosis. However, the expression throughout the cell cycle rules out a major regulatory role for cyclin A1 in the mitotic cell cycle. The promoter of cyclin A1 is dependent on four Sp1 transactivation sites in a CpG island upstream of the transcriptional start site. (89) Cyclin A1 is supposed to play a role in the pathogenesis of myeloid leukemia, since it is highly expressed in leukemias of myeloid origin. (90) Upon induction of myeloid differentiation cyclin A1 expression decreases.

(90) Overexpression of murine cyclin A1 in transgenic mice leads to abnormal myelopoiesis in the first month of the birth as well as to the development of myeloid leukemia at a low frequency. This indicates that cyclin A1 alone is not sufficient to induce transformation but contributes to leukemogenesis. (91)

In cancer tissue, elevated levels of cyclin A1 expression have been implicated in acute myeloid leukemia (86), acute lymphoblastic leukemia (92), biphenotypic acute leukemia (93) and also found in male germ cell tumor.(94) In solid tumor tissue, cyclin A1 was found to be highly expressed in poorly differentiated prostate cancer (92)and p53-apoptosis sensitive bladder cancer cell line.(95) In HeLa cells which is known HPV 18 infected cervical cancer cell lines was reported expression of cyclin A1 was detected by RT-PCR technique but not by northern blot technique. (81)

In normal tissue, the methylation of cyclin A1 promoter was detected in kidney, colon, spleen, testis, colon and small intestine, but not in brain, liver, pancreas or heart. (Figure 4). Expression of cyclin A1 was present in spleen, prostate, leukocyte, colon and thymus (Figure 5) by real time RT-PCR technique. (96)

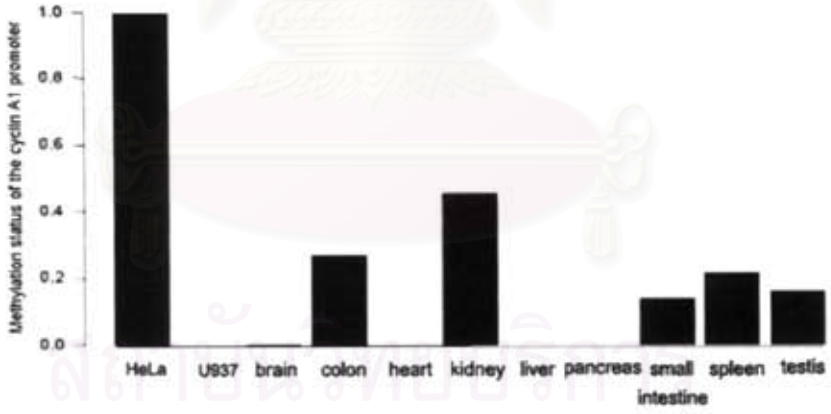


Figure 5. *CCNA1* promoter methylation status in human organs. The methylation status of the *CCNA1* promoter was analyses in the different human organ by real-time PCR technique. The degree of CpG methylation was calculated according to the formula Fraction of methylated molecules= 2 (threshold non-specific primers minus threshold methylation sqecific primers)

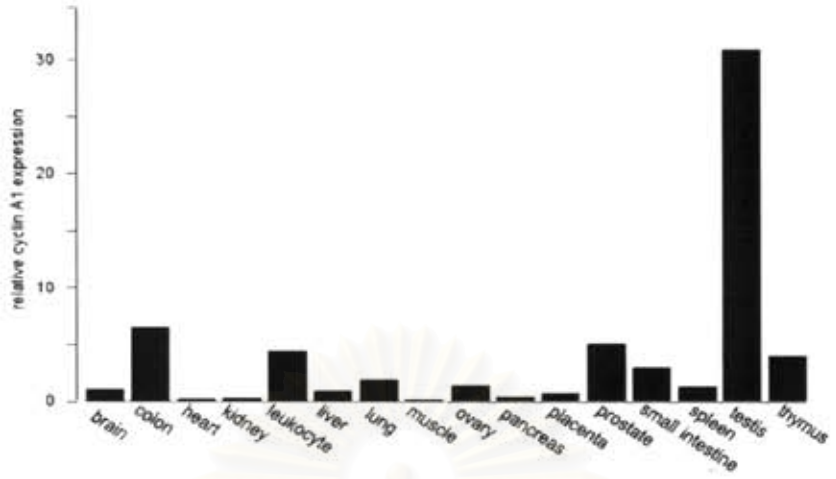


Figure 6. Cyclin A1 expression in human organs. Expression levels of *CCNA1* were analyzed in a panel of different human cDNAs by real-time quantitative PCR. Expression levels were standardized using expression of the house keeping gene *GAPDH*.

The interesting function of cyclin A1 is that it involved in DNA double strand break repair. After irradiation, cyclin A1 was induced by p53 on the transcriptional level. In addition, they identified the Ku70 DNA repair protein as a binding partner and substrate of the cyclin A1- CDK2 complex. DNA double strand break repair was deficient in *CCNA1*$^{-/-}$ cells. They found that both cyclin A1 and cyclin A2 enhance DNA double strand break repair by homologous recombination, but only cyclin A1 significantly activated nonhomologous end joining. (97)

Retinoic acid receptor responder1 (*RARRES1)*

Retinoic acid receptor responder1 (*RARRES1)* which acts as tumor suppressor gene, is a retinoid regulated gene. Its expression is frequently down regulated through DNA methylation in several types of malignant tissues. There is the study showed that loss of *TIG1* expression was strongly associated with *TIG1* promoter hypermethylation in leukemia, head and neck cancer, breast, colon, skin, brain, lung, and prostate cancer.

(98) Another group studied about the correlation between *TIG1* promoter hypermethylation and gene silencing in NPC. They showed that promoter hypermethylation is the major mechanism for *RARRES1* silencing. (99) The other group found that Tazarotene inducing gene (*TIG1)* methylation represented a new molecular marker for targeting diagnostic and therapeutic approaches in head and neck cancer.(100) Moreover, there was one group suggested that gastric carcinogenesis involved transcriptional inactivation by aberrant DNA methylation of *RARRES1*. (101) The other group reported that DNA methylation of *TIG1* gene was significantly associated with reduced expression of the respective mRNA. (102)

H-ras like suppressor 3 (*HRASLS3)*

Rat *HRASLS3* was identified as a gene down-regulated in *HRAS* transformed fibroblasts, and up-regulated in revertant and transformation-resistant fibroblasts. (103) Further investigation demonstrated that HRASLS3 possesses growth and tumor-inhibitory capacity and, therefore, belongs to the class II tumor suppressors. (104) interestingly, similar to the lysyl oxidase gene, down-regulation of *H-rev107* was found to be reversible. Recovery of its expression was obtained in KRAS transformed rat ovarian surface epithelial cells after inhibition of Mitogen-activated protein/Extracellular signal-regulated protein kinase (MAP/ERK) signalling pathway. (105) The human *HRASLS3* gene and its related gene *H-REV107-2/TIG3/RIG1* were cloned several years ago. (106) Both were demonstrated to possess transformation suppressive properties. H-REV107-1 was shown to be implicated in IFN signaling, and its expression was recovered in ovarian carcinoma cells after induction with IFN. (105) In the meantime it is known that an HRASLS3-like subfamily of proteins is exists, and consists of 5 members, which were demonstrated to be down-regulated in various human tumors. Their functions have not yet been elucidated in much detail, but resent phylogenetic analysis of the NlpC/P60 protein hydrolases demonstrated that HRASLS3-like proteins belong to this superfamily. (107)

Karin Roder, *et al.* studied about correlation between gene silencing and promoter hypermethylation of this gene in WEHI7.1 lymphoma cells. They found that hypermethylation led to loss of expression of HRASLS3. (108) Recently, Irina Nazarenko, *et al* reported the opposite function of this gene. They found that sixty eight percent of lung tumors revealed positive HRASLS3 specific staining. Furthermore, survival analysis demonstrated a significant association of cytoplasmic HRASLS3 with decreased patient survival. This suggested that *HRASLS3*, which also known as a tumor suppressor gene, play a different role in non-small cell lung carcinoma by contributing to tumor progression. (109)

CHAPTER III

MATERIAL AND METHOD

1. Sample

Tumor samples

Primary NPC tumor samples were surgically obtained from patients with appropriate informed consent from The King Chulalongkorn Memorial Hospital. Samples were then divided into 2 portions. The first portion was fixed in formalin and was submitted for routine histo-pathological examination, and the second portion was immediately snap-frozen and stored in liquid nitrogen until use in further experiments. Control nasopharyngeal epithelium was obtained from nasopharyngeal swabs from unrelated patients in the Department of Otolaryngology who had clinically defined normal nasopharyngeal mucosa. Control blood samples were additionally collected from unrelated healthy donors. All samples were tested to confirm the presence of the genetic material of EBV following the method previously reported.

Cultured NPC cells

Primary NPC tissue was finely chopped, suspended in 0.25% collagenase(Gibco BRL Gaithersberg, MD, USA) and incubated at 37°C, 5% CO_2 for 4-5 hours. After centrifugation, the collagenase was discarded and the pellet was subjected to two washing steps with 5 ml DMEM (Dulbecco's Modified Eagle Medium; Gibco) each. Subsequently, the pellet was resuspended in 7 ml DMEM devoid of both growth factors and fetal calf serum, with 1% penicillin/streptomycin added and incubated at 37°C, 5% CO_2 until the resulting cell layer had spreaded sufficiently to be passaged. Pellets of NPC cells with limited passages were collected for DNA extraction.

Presence of NPC cells was confirmed by the detection of LOH of chromosome 3, 9, and 14, using microsatellite analysis for the following markers; D3S1038, D14S283 and TCRD, in order to confirm the complete loss of heterozygosity as well as to show the

absence of contaminating normal allele. Briefly, one strand of each primer pair was end-labeled at 37°C for 1 to 2 hr in a total volume of 10 μl containing10 μM primer, 0.025 mCi [^{32}P] γATP (Amersham, Aylesbury, UK) at 3,000 Ci/mmol, 10 mM $MgCl_2$, 5 mM DTT, 70 mM Tris-HCl (pH 7.6) and 10 units of T4 polynucleotide kinase (New England Biolabs, Beverly, MA). Without further separating the unincorporated nucleotides, the kinase reaction was added to the PCR buffer mix. The PCR reactions were performed in a total volume of 10 μl using 50 ng of genomic DNA in 200 μM dNTP each, 10 mM Tris-HCL (pH 8.4), 50 mM KCl, 1.5 mM MgCl2, 0.5 units of *Thermus aquaticus* DNA polymerase (Perkin Elmer Cetus, Norwalk, CT) and primer concentrations were 0.05 to 0.5 μM each. Several PCR reactions were optimized as follows: an initial de-naturation step at 95° C for 4 min, followed by 25 cycles of de-naturation at 94° C for 1 min, annealing at 55° C for 1 min, extension at72° C for 2 min and a final extension at 72° C for 7 min. Aliquots (2 μl) of each reaction were mixed with 1 μl formamide loading buffer, heated at 95° C for 2 min, put on ice for 30 sec, then loaded onto 6% polyacrylamide 7 M urea gel.

2. Materials

1.1 E. coli (DH5α)

1.2 pGEM-T easy vector

1.3 DNA purification kit (Promega, USA)

1.4 DNA clean up kit (Promega, USA)

1.5 MicroAmp PCR tube

1.6 Cryotube (Nunc, USA)

1.7 Counting chambers

1.8 Pipette tip : 10 μl, 100 μl,1,000 μl (Elkay, USA)

1.9 Microcentrifuge tube : 0.2 ml, 0.5 ml, 1.5 ml (Bio-rad Elkay, USA)

1.10 Beaker : 50 ml, 100 ml, 200 ml, 500 ml, 1,000 ml (Pyrex)

1.11 Flask : 250 ml, 500 ml, 1,000 ml (Pyrex)

1.12 Reagent bottle : 100 ml, 250 ml, 500 ml, 1,000 ml (Duran, USA)

1.13 Cylinder : 25 ml, 50 ml, 100 ml, 250 ml, 500 ml, 1,000 ml (Witeg, Germany)

1.14 Glass Pipette : 5 ml, 10 ml (Witeg, Germany)

1.15 Microcentrifuge tube rack (Scientific plastics, USA)

1.16 Thermometer (Precision, Germany)

1.17 Plastic wrap

1.18 Stirring-magnetic bar

2. Equipments

2.1 Light microscope

2.2 Stereo microscope

2.3 Autoclave

2.4 Microwave oven

2.5 Hot air oven (Memmert, West Germany)

2.6 Pipette boy (Tecnomara, Switzerland)

2.7 Vortex (Scientific Industry, USA)

2.8 pH meter (Eutech Cybernatics)

2.9 Stirring hot plate (Barnstead/Thermolyne, USA)

2.10 Balance (Precisa, Switzerland)

2.11 Microcentrifuge (Fotodyne, USA)

2.12 DNA Thermal cycler 480 (Perkin Elmer, Cetus USA)

2.13 Thermal cycler (Touch Down, Hybraid USA) Power supply model 250 (Gibco BRL, Scothland)

2.14 Power poc 3000 (Bio-Rad, USA)

2.15 Horizon 11-14 (Gibco BRL, Scothland)

2.16 Beta shield (C.B.S scientific. Co.)

2.17 Heat block (Bockel)

2.18 Incubator (Memmert, West Germany)

2.19 Thermostat shaking-water bath (Heto, Denmark)

2.20 Spectronic spectrophotometers (Genesys5, Milon Roy USA)

2.21 UV Transilluminator (Fotodyne USA)

2.22 UV-absorbing face shield (Spectronic, USA)

2.23 Gel doc 1000 (Bio-RAD)

2.24 Refrigerator 4 ^{0}C (Misubishi, Japan)

2.25 Deep freeze -20 ^{0}C, -80 ^{0}C (Revco)

2.26 Water purification equipment (Water pro Ps, Labconco USA)

2.27 Water bath (Memmert, West Germany)

2.28 Storm 840 and ImageQuaNT software (Molecular dynamics)

2.29 Gel star nucleic acid gel stain (Cambrex Bio Science)

3. Reagents

1 General reagent

1.1 Absolute ethanol (Merck)

1.2 Agarose gel (FMC Bioproducts)

1.3 Ammonium acetate (Merck)

1.4 Bisulfite (Merck)

1.5 Bromphenol blue (Pharmacia)

1.6 Chloroform (Merck)

1.7 Disodium ethylenediamine tetracetic acid : EDTA (Merck)

1.8 Dimethyl sulfoxide (DMSO) (Sigma)

1.9 Dnasel (Gibco BRL)

1.10 Diethylpyrocarbonate(DEPC) (Sigma) Ethidium bromide (Gibco BRL)

1.11 Fetal bovine serum (Gibco BRL)

1.12 Guanidium thiocyanate (USB)

1.13 Glycogen (Sigma)

1.14 Hydrochloric acid (Merck)

1.15 Hydroquinone (Merck)

1.16 IPTG (Promega)

1.17 Isoamyl alcohol (Merck)

1.18 Isopropanol (Merck)

1.19 LB medium (Gibco BRL)

1.20 Mineral oil (Sigma)

1.21 Phenol (Sigma)

1.22 Penicillin/Streptomycin (Gibco BRL)

1.23 Sodium acetate (Sigma)

1.24 Sodium bisulfite (Sigma)

1.25 Sodium chloride (Merck)

1.26 Sodium hydroxide (Merck)

1.27 Trypan blue (Gibco BRL)

1.28 Triton X-100 (Pharmacia)

1.29 Trizoll reagent (Invitrogen)

1.30 X-gal (Promega)

1.31 Xylene (Merck)

1.32 10 base pair DNA ladder (Biolabs)

1.33 40% acrylamide/bis solution 19:1 (Bio-Rad)

1.34 Wizard DNA Clean-up System(Promega)

1.35 QIAamp DNA blood mini kit(QIAGEN)

1.36 2-Mercaptoethanol (2-ME) (Sigma)

2. Reagent of PCR

2.1 10X PCR buffer (500 mM KCl, 200 mM Tris-HCl pH 8.4) (GibcoBRL, Perkin Elmer)

2.2 Magnesium chloride (GibcoBRL, Perkin Elmer)

2.3 Deoxynucleotide triphosphates (dNTPs) (Promega)

2.4 Oligonucleotide primers (BSU, GENSET) in appendix B

2.5 Hotstart*Taq* DNA polymerase (Qiagen)

2.6 Genomic DNA sample

4. Methods

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DNA extraction

Extraction of genomic DNA was performed with proteinase K (Amersham, Aylesbury, UK) digestion in the presence of SDS at 50°C overnight, followed by phenol/chloroform extraction and ethanol precipitation.

White blood cells (WBC)

Five to ten ml of whole blood was centrifuged for 10 min at 1500 g. The supernatant was removed and the buffy coat was collected to a new polypropylene tube. The buffy coat was added with 10 volumes of cold lysis buffer 1 (or 10 ml), then was mixed thoroughly and incubated at -20°C for 5 min. The tube was centrifuged for10 min at 1000 g, then the supernatant was removed. The pellet was added with 3 ml of cold lysis buffer 1, then was mixed thoroughly and was centrifuged at 1000 g for 5 min. The supernatant was discarded and the pellet was added with 900 µl of lysis buffer 2, 10 µl of Proteinase K solution (20 mg of Proteinase K in 1.0 ml of 1% SDS EDTA, prepared 30 min before use), and 50 µl of 10% SDS, then was mix vigorously for 15 seconds. The sample was incubated in water bath at 37°C overnight (16-24 h) for complete digestion. The sample was added with 1 volume of phenol-chloroform-isoamyl alcohol (25:24:1),

shaked vigorously for15 seconds and then was centrifuged at 10,000 g for 5 min. The supernatant was transferred to a new microcentrifuge tube. The DNA was precipitated with 0.5 volume of 7.5 M Ammonium acetate (CH_3COONH_4) and 1 volume of cold (100%) absolute ethanol and then were mixed by inversion. The DNA should immediately form a stringy precipitate. The DNA was then recovered by centrifugation at 10,000 g for 15 min. The supernatant was removed and the pellet was washed with 1 volume of 70% ethanol and centrifuged at 7,500 g for 5 min. The ethanol was decanted and the pellet was air dried. The digested DNA was re-suspended in 50 µl of the double distilled water at 65 °C until dissolved. The DNA was stored at -70 °C until the DNA methylation assay was performed.

Bisulfite Treatment of DNA

Freshly prepared 10 mM Hydroquinone (Sigma: H-9003) and 3M Sodium bisulfite (Sigma: S9000)

- Add 55 mg of Hydroquinone to 50 ml of dH_2O (for Deamination).
- Add 3.76 mg of Sodium bisulfite (for Sulphonation) to 10 ml of dH_2O and adjust pH to 5 with NaOH.

DNA preparation

1. Dilute DNA 1-2 µg with 50 µl distilled H_2O in a total volume of 50 µl

   Example DNA 1000 ng/ul

   1000 ng ------ 1 ul

   2000 ng (2ug) ------ $\frac{2000 * 1}{1000} = 2.00$ µl

   Pipette DNA 2 ul and add dH_2O 48 ul

2. Add 5.5 ul of 2M NaOH, mix and incubate at 37 °C in water bath for 10 minutes (to create single stranded DNA).

3. Add 30 ul of 10 mM Hydroquinone and 520 ul of 3M Sodium bisulfite to each tube, after mixing, spin down and incubate at 50 ^{0}C in water bath for 16 hours.
4. Prepare Vac-Man® Laboratory Vacuum Manifold (Promega A7231).
5. Add 1 ml of Wizard DNA clean-up Resin (Promega A7280) to each tube and transfer the mixture to Syringe Barrels. After that turn on the pump until the mixture in the column become dry.
6. Wash with 2 ml of 80% isopropanol and turn on the pump until the mixture in the column is dry.
7. Turn on the pump for 30 seconds to the mixture.

8. Place minicolumns in clean tubes and centrifuge at 10,000g (RCF) for 2 min.
9. Add 50 ul of heated water (at 95 ^{0}C) and incubate at room temperature for 1 min.
10. Spin down at 10,000g (RCF) for 20 seconds.
11. Add 5.5 ul of 3M NaOH to each tube and incubate at room temperature for 5 min.
12. Add 1 ul of 20 ng/ul Glycogen (as carrier).
13. Add 17 ul of 10M NH_4Ac and 3 volumes of ice-cold 100% Ethanol.
14. Mix and incubate at -20 ^{0}C for 2 hours.
15. Centrifuge at 14,000 rpm for 15 min.
16. Discard supernatant and wash pellet with 200 µl of70% ethanol (ice-cold).
17. Mix and centrifuge at 14,000 rpm for 10 min.
18. Discard supernatant and air dry.
19. Resuspend DNA in 20 µl of dH_2O and incubate at 37 ^{0}C for 10 min.
20. The bisulfite DNA can be used for PCR.

Primer design for eight candidate genes

Primers for Methylated specific PCR (MSP)

Primers were designed to amplify the methylated and unmethylated alleles equally. The primer design was base on the difference between methylated allele and non-methylated allele after standard sodium bisulfite treatment. PCR amplification results in the conversion of unmethylated cytosine residues to thymine and methylated cytosine residues to cytosine. The sequence conversion leads to the methylation-dependent creation of specific PCR amplification which is different between methylated and non-methylated allelle. The general strategy is shown below:

- Identify the 5′UTR of genes / CpG islands. This is the unconverted map.
- Copy sequence and paste in a text editor.
- Convert all C to T except for CG. First convert all CG to XG. Then convert all C to T. Then convert all X to C. Make a map of this converted sequence (methylated map)
- Convert all remaining C to T. Make a map of this converted sequence(Non-methylated map)
- Design 2 pairs of primers covering the CG rich region using methylated and unmethlated maps. All primer sequences were shown in table1 and figure 7

A

Original : *CCNA1*

ccagcgtgggcagggcgccgcagcctgcgcagccccgaggaccccgcgtcgctctcccgagccagggttctcaggagc

Met:

TTagcgtgggTagggcgTcgTagTTtgcgTagTTTcgaggaTTTcgcgtcgTtTtTTcgagTTagggttTtTaggagc

Unmet:

TTagTgtgggTagggTgTTgTagTTtgTgTagTTTTgaggaTTTTgTgtTgTtTtTTTgagTTagggttTtTaggagT

Original: *EML1*

cacgtccccctcccggcccgggccccgcggccgccgaggccgccccctcgcgggcggagcgggcgctgggctcgcgcggctgcggcggcggcggcgggcccggggttgccatggtaaccggcagcagcagccgccacagcagggccggcccacgcgccagcgccggtcgcggtcgggctcagctcagtgtgtggtgagcggcggcggcgcggccgggccggggagcgggcgcggcccggc

Met:

TacgtTTTTTtTTcggTTcgggTTTcgcggTcgTcgaggTcgTTTTTtcgcgggcggagcgggcgTtgggTtcgcgcggTtgcggcggcggcggcgggTTcggggttgTTatggtaaTcggTagTagTagTcgTTaTagTagggTcggTTTTagcgTTagcgTcggtcgcggtcgggTtTagTtTagtgtgtggtgagcggcggcggcgcggTcgggTcggggagcgggcgcggTTcggT

Unmet:

TaTgtTTTTTtTTTggTTTgggTTTTgTggTTgTTgaggTTgTTTTTtTgTgggTggagTgggTgTtgggTtTgTgTggTtgTggTggTggTggTgggTTTggggttgTTatggtaaTTggTagTagTagTTgTTaTagTagggTTggTTTTagTgTTagTgTTggtTgTggtTgggTtTagTtTagtgtgtggtgagTggTggTggTgTggTTgggTTggggagTgggTgTggTTTggT

Original: *CLMN*

cacgtccccctcccggcccgggccccgcggccgccgaggccgccccctcgcgggcggagcgggcgctgggctcgcgc
ggctgcggcggcggcggcgggcccggggttgccatggtaaccggcagcagcagccgccacagcagggccggcccca
gcgccagcgccggtcgcggtcgggctcagctcagtgtgtggtgagcggcggcggcgcggccgggccggggagcgggc
gcggcccggc

Met:

TacgtTTTTTtTTcggTTcgggTTTcgcggTcgTcgaggTcgTTTTTtcgcgggcggagcgggcgTtgggTt
cgcgcggTtgcggcggcggcggcgggTTcggggttgTTatggtaaTcggTagTagTagTcgTTaTagTagggT
cggTTTTagcgTTagcgTcggtcgcggtcgggTtTagTtTagtgtgtggtgagcggcggcggcgcggTcgggTcg
gggagcgggcgcggTTcggT

Unmet:

TaTgtTTTTTtTTTggTTTgggTTTTgTggTTgTTgaggTTgTTTTTtTgTgggTggagTgggTgTtgg
gTtTgTgTggTtgTggTggTggTggTgggTTTggggttgTTatggtaaTTggTagTagTagTTgTTaTagT
agggTTggTTTTagTgTTagTgTTggtTgTggtTgggTtTagTtTagtgtgtggtgagTggTggTggTgTgg
TTgggTTggggagTgggTgTggTTTggT

Original: *TSC22*

gcatgcccagtagctcagacggttcggttacaaataggaagctttcgccgttaagcagccgcggggacacagctccgggg
accggggtctgactcccttaaacgtaagcacgtgtgggggcccccatagtatttttcagggacccttcacattttctgccgaag
caatggcccagtgttttgaggccgccgtggaaacctccgaacccagcaccccactccctctgagcacggcccgcgccag
gccagcccaccccacgagcgggatctggagaatgggggcggaggtgggggtgggggaggcggggaagcagggaa
gcgggaggacctggaaggagtagggggcggtcccagtggcgtgccggccaatcggcgcactctttcctgcatatattttgc
actaagactgggagtcctggtctagagctgagtggagcccg

Met:

gTatgTTTagtagTtTagacggttcggttaTaaataggaagTtttcgTcgttaagTagTcgcggggaTaTagTtTcg
gggaTcggggtTtgaTtTTTttaaacgtaagcacgtgtgggggTTTTTatagtatttttTagggaTTTttTaTattttTt
gTcgaagTaatggTTTagtgttttgaggTcgTcgtggaaaTTtTcgaaTTTagTaTTTTaTtTTTtTtgagTac
ggTTcgcgTTaggTTagTTTaTTTTacgagcgggatTtggagaatgggggcggaggtgggggtgggggaggg
cggggaagTagggaagcgggaggaTTtggaaggagtagggggcggtTTTagtggcgtgTcggTTaatcggcgT
aTtTtttTTtgTatatattttgTaTtaagaTtgggagtTTtggtTtagagTtgagtggagTTcg

Unmet:

gTatgTTTagtagTtTagaTggttTggttaTaaataggaagTtttTgTTgttaagTagTTgTggggaTaTagTtTT
ggggaTTggggtTtgaTtTTTttaaaTgtaagTaTgtgtgggggTTTTTatagtatttttTagggaTTTttTaTatttt
TtgTTgaagTaatggTTTagtgttttgaggTTgTTgtggaaaTTtTTgaaTTTagTaTTTTaTtTTTtTtgagT
aTggTTTgTgTTaggTTagTTTaTTTTaTgagTgggatTtggagaatgggggTggaggtgggggtgggggg
aggTggggaagTagggaagTgggaggaTTtggaaggagtagggggTggtTTTagtggTgtgTTggTTaatTg
gTgTaTtTtttTTtgTatatattttgTaTtaagaTtgggagtTTtggtTtagagTtgagtggagTTTg

B

HRASLS3

Before bisulfite :

ggtgcctgggatgcttctcccccttccgcgaggaagagatctaattgggtagggcgggtgtagactagcctgccgagccgcc
cgctggcacctgcagcctcctgggcgcc

After bisulfite:

ggtgTTtgggatgTttTtTTTTttTcgcgaggaagagatTtaattgggtagggcgggtgtagaTtagTTtgTcgagTc
gTTcgTtggTaTTtgTagTTtTTtgggcgTT

LOH11CR2A

Before bisulfite:

ctgaggaaatgaggctgtaagcctcatctactccaatcagttgcagcccctaag
cccttctctatagaaatgtaagagaccgacgcagtctcttctgtaaatacgtggacattg
gaaatctaggact

After bisulfite:

TtgaggaaatgaggTtgtaagTTtTatTtaTtTTaatTagttgTagTTTTtaag
TTTttTtTtatagaaatgtaagagaTcgac gTagtTtTttTtgtaaatac gtggaTattg
gaaatTtaggaTt

MCC

Before bisulfite:

aaaatgtggcagaagggaccaagcagtggatattgagcctgtgaagtccaactcttaagctccgagacctgggggactga
gagccc

After bisulfite:

aaaatgtggTagaagggaTTaagTagtggatattgagTTtgtgaagtTTaaTtTttaagTtTcgagaTTtgggggga
TtgagagTTT

RARRES1

Before bisulfite:

gggaggCgtccccattgtcccccccaacccCgaccctctccCgTCgCggcctgggccagaagcaccCggccctgCgc
tgCggaggCgatgcCgcatcctagcactaagccCgggcag

After bisulfite:

gggaggCgtTTTTattgtTTTTTTaaTTTCgaTTTtTtTTCgTCgCggTTtgggTTagaagTaTTCggTT
TtgCgTtgCggaggCgatgTCgTatTTtagTaTtaagTTCgggTag

Figure 7: Bisulfite modification sequence and primer regions. A: diagram of methylated and non-methylated sequences after bisulfite modification covering the area of both primers (underlined) in the promoter region of *CCNA1,EML1,CLMN* and *TSC22*, B: diagram of methylated sequence after bisulfite modification covering TCGA sequence, (which a recognition site for*Taq1* cut)in the promoter region of *HRASLS3, LOH11CR2A, MCC* and *RARRES1*

Primers for combined bisulfited restriction analysis (COBRA)

At the beginning, CG at the promoter of genes must be converted to TG. *Taq1* restriction enzyme which recognizes TCGA sequence was used in this study for detecting methylation. The sequences without TG (T comes from C) around TCGA were design for primer. All primer sequences were shown in table1 and figure 6.

Methylation detection

Duplex MSP and COBRA

The methylation status of *CCNA1*, *TSC22*, *CLMN* and *EML1* was performed by the duplex MSP whereas that the methylation status of *HRASLS3*, *RARRES1*, *LOH11CR2A* and *MCC* was performed using the combined bisulfite restriction analysis (COBRA). The PCR reactions contained 1x PCR buffer (Qiagen, Chuo-ku), 0.2 mM deoxynucleotide triphosphates, 0.4 μM of appropriate PCR primers as shown in table2, 1 U of hotstarTaq (Qiagen, Chuo-ku), and 80 ng of bisulfited DNA. The amplification reactions were performed in a thermal cycler. PCR reactions have been optimized as follow: an initial de-naturation step at 95° C for 10 min, followed by 30 cycles of de- naturation at 95° C for 1 min, annealing at 48-55 ° C 1 min (as described in table1), extension at 72° C for 2 min and a final extension at 72° C for 5 min. In the COBRA analysis, all PCR products were digested with *Taq1* enzyme and subjected to electrophoresis in 8% native polyacrylamide gel. The gel was visualized using phosphoimager after staining with cyber green.

Bisulfite genome sequence analysis

Some *CCNA1*, *RARRES1* and *HRASLS3* methylation-positive NPC were selected for sequence analysis. The bisulfited DNAs were amplified using CCNA1cloningF and CCNA1cloningR (Table1). The amplified fragments were cloned using the pGem-T easy vector (Promega) according to the manufacturer's protocol. The ligated products were transformed into *E. coli* DH5α. The clones were selected by X-gal/IPTG and amplicillin.

Recombinat plasmids were purified by DNA purified PCR kit(Promega) according to the manufacturer's protocol. For sequencing analysis, the plasmids were used for the sequencing reaction using Prism Ready Reaction DyeDeoxy Terminator FS cycle Sequencing Kit (Applied Biosystem) according to the manufacturer's instruction. The DNA template was mixed with 8 µl of Prism Terminator Mix, 3.2 pmol of primer M13 (sense). The distilled water was added to bring the final volume to 20 µl reaction. The sequencing reaction was subjected to 25 PCR cycles, each consisted of 96 °C for 30 seconds, 50 °C for 15 seconds and 60 °C for 4 min in a thermalcycler (GeneAmp PCR system 2400)

The sequencing reaction was purified using simplified ethanol precipitation. Ten microliters of sequencing reaction were added with 2 µl of 3M Sodium acetate (NaOAc), 50 µl of 95% ethanol, vortexed briefly, and left stand at room temperature in the dark of 15 min. The precipitate of sequencing reaction was centrifuged at 13,000 g at room temperature for 20 min, and the pellet was washed with 250 µl of 70% ethanol. The suspension was centrifuged at 13,000 g at room temperature for 5 min, and the pellet was dried for 1 min at 90 °C in a Dri-bath . The pellet could be kept at -20 °C for 1 week.

The pellet was subjected to sequencing analysis by ABI Prism 310 Genetic Analyzer (PE-Applied Biosystems). Regarding the rest of subsequent steps, we referred to the ABI Prism 310 Genetic Analyzer user's manual. The nucleotide sequences were analyzed with Sequence Analysis Software which analyzed the electropherogram pattern in comparision with the de matrix file.

Statistical Analysis

Fisher's exact and chi square tests were used to determine the association between clinical parameters and the presence of promoter hypermethylation of the studied genes.

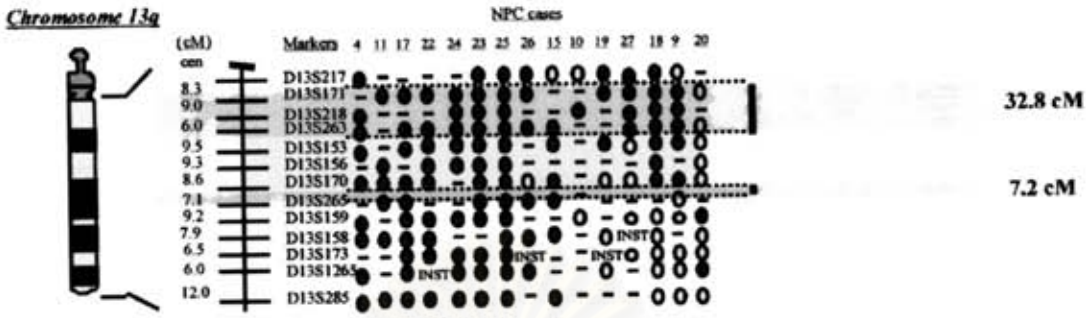
CHAPTER IV

RESULTS

Selection of tumor suppressor gene candidates from the genome-wide expression analysis of NPC.

At the beginning, a list of genes whose expression was shown to be differentially down-regulated in NPC when compared to the normal nasopharyngeal epithelium was retrieved. To select for the tumor suppressor candidates, we applies 2 additional criteria. These include genes that have previously been shown to be tumor suppressor genes in other cancer types as well as genes residing within the critical region of LOH in chromosome 13 and 14, which are also thought to be important areas in NPC. From the 238 down-regulated genes in NPC, 4 genes that were indicated to be tumor suppressor genes in other cancer were selected; *HRASLS3*, *RARRES1*, *LOH11CR2A* and *MCC*. Furthermore, *CCNA1* and *TSC22* whose genetic locations are within the critical LOH region of chromosome 13 at position 35948-35949 kb and 44010-44020 kb, respectively were chosen. (17) Finally, *EML1* and *CLMN* which resides within the critical LOH region on chromosome 14 at position 98320-98330 kb and 93710-93720 kb, respectively were selected due to their low expression in NPC. The location of these 4 genes in critical LOH region was shown in figure 7and 8.

A



B

Markers and Genes	Location (kb)
D13S171	32151-32152
CCNA1	35948-35949
D13S218	37930
D13S263	40978-40979
TSC22	44010-44020
D13S153	47788
D13S266	88322

Figure 7 : A showed the LOH critical region from the previous study on chromosome 13 (pink highlight show the critical region), B showed the location of interesting genes and markers in LOH critical region.

A

B

Markers and Genes	Location (kb)
D14S280	91252
CLMN	93710-93720
D14S65	96691
EML1	98320-98330
D14S985	100366
D14S292	103666

Figure 8 : A showed the LOH critical region from the previous study on chromosome 14 (pink highlight show the critical region), B showed the location of interesting genes and markers in LOH critical region.

Analysis of the methylation status of promoter regions of candidate genes

The potential mechanism which might be responsible for the down-regulation of all eight candidate genes was further investigated. The 5' elements of all eight genes were analyzed for methylation status by either MSP or COBRA technique as shown in table 1 in several NPC tissues and control normal peripheral blood leukocytes. All NPC were positive for EBV while the control tissues were negative. Hypermethylation was detected on the *CCNA1*, *RARRES1* and *HRASLS3* promoter in NPC but not in the control WBC (Fig 10, 11 and table 2). However, there was no methylation detected on the *MCC*, *TSC22*, *EML1* and *CLMN* regulatory region. Partial methylation of LOH11CR2A promoter could be observed in both NPC and control WBC (Fig10,11 and table 2).Our findings in conjunction with the microarray data suggest thatthe promoter hypermethylation may be crucial in regulating expression of *CCNA1*, *RARRES1* and *HRASLS3* in NPC.

Promoter hypermethylation of *CCNA1*, *RARRES1* and *HRASLS3*

To further validate the crucial role of promoter hypermethylation of these tumor suppressor gene candidates in NPC carcinogenesis, the frequency of methylation of *CCNA1*, *RARRES1* and *HRASLS3* in approximately 100 NPC tissue samples either by COBRA or MSP PCR. Additional 10 primary cultures of NPCs to isolate an enriched sub-set of tumor cells were also analyzed for the methylation status of these genes. Several controls used in this study were 20 normal nasopharyngeal swabs and 30 WBC samples from healthy subjects or non cancer patients. Strikingly, in all normal cells including nasopharyngeal epithelium and WBC, the presence of promoter hypermethylation of all the studied genes was not demonstrated. In contrast, NPC tissues displayed frequent promoter hypermethylation for 57% of *CCNA1*, *54%* of *RARRES1* and 24% of *HRASLS3* (Table 3). Moreover, the frequent promoter hypermethylation in isolated NPC cells (to avoid normal cell contamination) was found 100% of *CCNA1*, 83% of *RARRES1* and 17% of *HRASLS3*. Additional analyses of the promoter sequence of all three genes to

prove the presence of promoter hypermethylation were performed as shown in figure 12 and 13.

***CCNA1*, *RARRES1* and *HRASLS3* methylation and Clinicopathlogical correlation**

The correlation between promoter hypermethylation and clinical parameters was further elucidated. We found no significant correlation with the tumor stage. (Table 4)

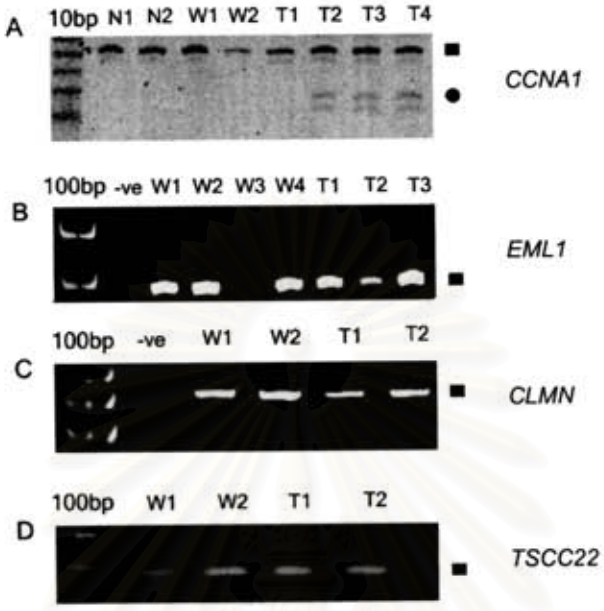


Fig 10. MSP analysis. (A) The results of an MSP analysis of *CCNA1* show frequent methylation of 5' element of *CCNA1* in NPC but not in normal leukocyte and nasopharyngeal epithelium. (B-D) The results of MSP analysis of *EML1*, *CLMN*, and *TSCC22*, are shown. There was no methylation observed in these sets of genes. Square: an unmethylated band, circle: a methylated band, -ve: control lane (water), T: NPC tissue, W: leukocytes, N: normal nasopharyngeal swab.

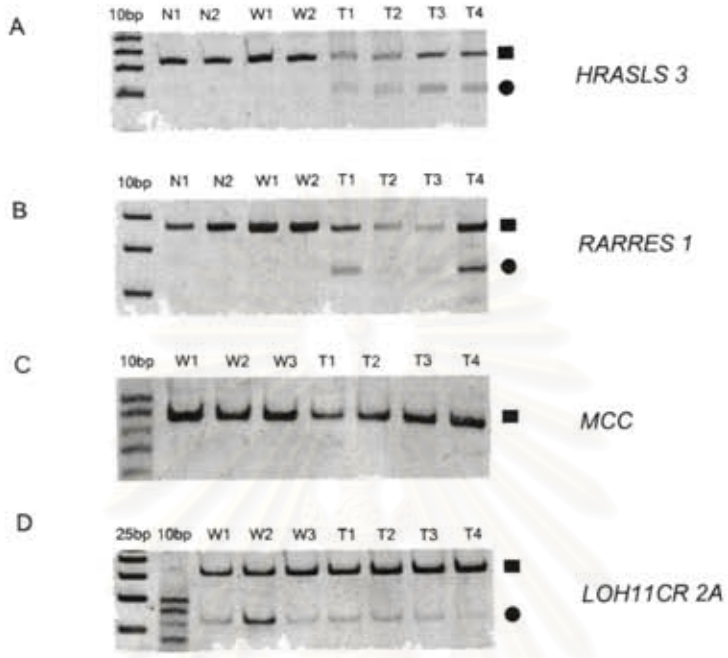
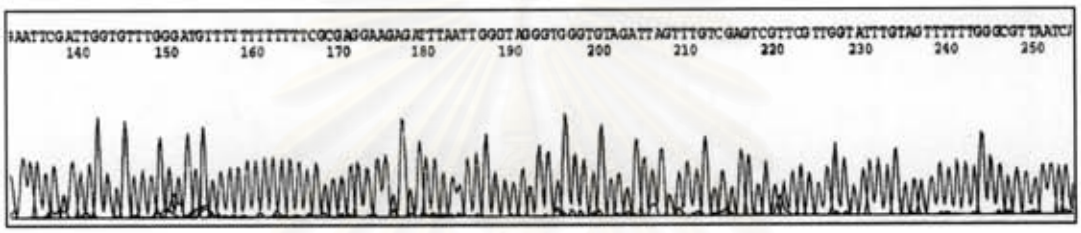


Fig 11. COBRA analysis. Methylation status of *HRASLS3*, *RARRES1*, MCC, and *LOH11CR* 2A promoter was analyzed by COBRA technique. Panel A and B showed frequent methylation of *HRASLS3* and *RARRES1* upstream elements in NPC but not in normal tissues. Methylation of *MCC* was not detected in both normal and tumor cells. In panel D, methylation of *LOH11CR2A* DNA sequence are invariably presence in both normal and tumor cells. Square: unmethylated band, circle: methylated band, T: NPC tissue, W: leukocytes, N: normal nasopharyngeal swab.

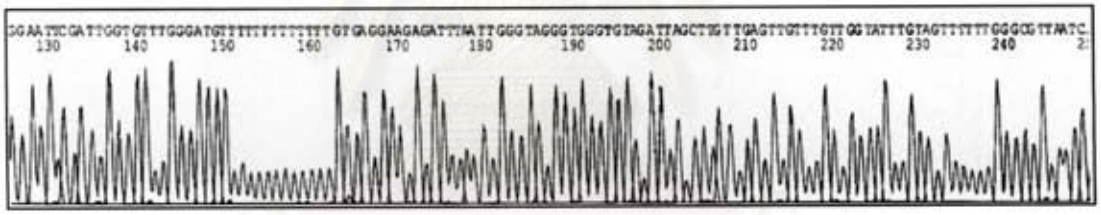
A *CCNA1* methylation

B *CCNA1* unmethylation

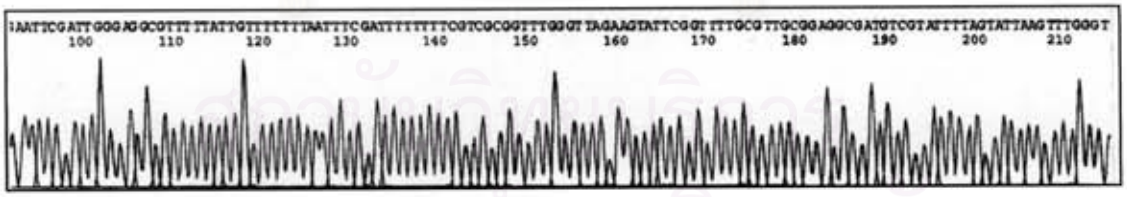
C: *HRASLS3* methylation



D: *HRASLS3* unmethylation



E: *RARRES1* methylation



F: *RARRES1* unmethylation

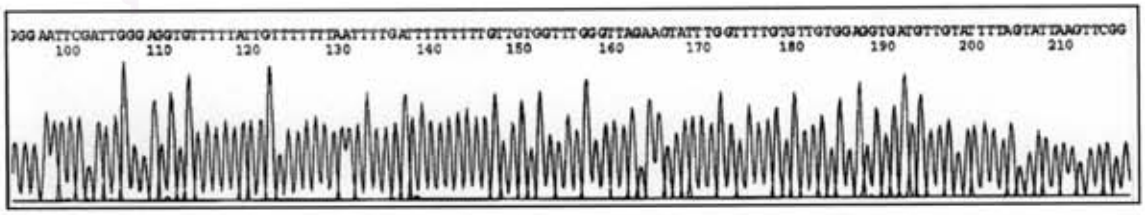
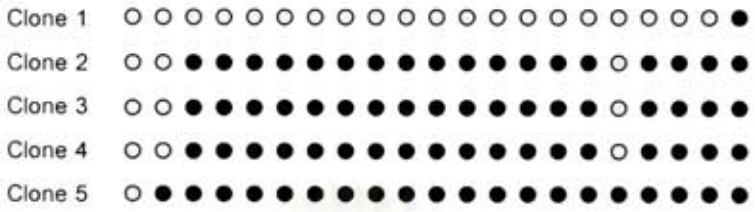


Figure 12: Sequence analysis of 3 candidate tumor suppressor genes, A: *CCNA1* promoter hypermethylation in NPC, B: *CCNA1* promoter unmethylation in normal cells, C: *HRASLS3* promoter hypermethylation in NPC, D: *HRASLS3* promoter unmethylation in normal cell, E: *RARRES1* promoter hypermethylation in NPC, F: *RARRES1* promoter unmethylation in normal cells.

A. *CCNA1*



B. *HRASLS3*

C. *RARRES1*

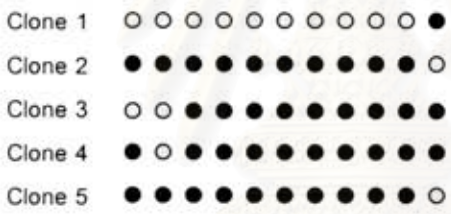


Figure 13: Diagram of bisulfite sequencing at promoter of candidate tumor suppressor genes in NPC; A: *CCNA1*, B: *HRASLS3* and C: *RARRES1* circles denote the methylation status of each selected clone. Black circles are methylated CG dinucleotides, and white circles are non-methylated CpG dinucleotides or TG dinucleotides.

Table 1: Selected candidate genes and primers used to detect methylation

gene	Detection	Primer	Sequence	Tm (°C)
CCNA1 [a]	MSP	*CCNA1* met (F)	TTTCGAGGATTTCGCGTCGT	53
		CCNA1 met (R)	CTCCTAAAAACCCTAACTCGA	
		CCNA1 unmet (F)	TTAGTGTGGGTAGGGTGTT	53
		CCNA1 unmet (R)	CCCTAACTCAAAAAAACAACACA	
TSCC22 [a]	MSP	**TSCC22 *met (F)***	TTTAGTGTTTTGAGGTCGTC	48
		TSCC22 met (R)	TAAAATAAACTAACCTAACGCGA	
		TSCC22 unmet (F)	GGGATATAGTTTTGGGGATT	48
		TSCC22 unmet (R)	ATAAACTAACCTAACACAAACCA	
CLMN [a]	MSP	*CLMN* met (F)	AAACCTAACTAACAAACGCG	55
		CLMN met (R)	TCGTATTCGTCGTTTCGC	
		CLMN unmet (F)	TGTTGTGATTTTAGTTTTGTGGT	55
		CLMN unmet (R)	CCAACACAACACAAACAACA	
EML1 [a]	MSP	*EML1* met (F)	TCGAGGTCGTTTTTTCGC	55
		***EML1* met (R)**	ACGCTAACGCTAAAACCG	
		EML1 unmet (F)	TTTGGGTTTTGTGGTTGT	55
		EML1 unmet (R)	ACCCAACCACAACCAACA	
HRASLS3 [b]	COBRA	*HRASLS3* (F)	GGTGTTTGGGATGTTTTTTT	55
		HRASLS3 (R)	AAC (A/G) CCCAAAAAACTACAAA	
MCC [b]	COBRA	*MCC* (F)	AAAATGTGGTAGAAGGGATT	53
		MCC (R)	AAACTCTCAATCCCCCAAA	
RARRES1 [b]	COBRA	*RARRES1* (F)	GGGAGG (C/T)GTTTTTATTGTTTT	53
		RARRES1 (R)	CTACCC (A/G)AACTTAATACTAAA	
LOH11CR2A [b]	COBRA	*LOH11CR2A* (F)	TTGAGGAAATGAGGTTGTAAGTT	48
		LOH11CR2A (R)	AATCCTAAATTTCCAATATCCAC	

Table 2: Product size of each primer

gene	MSP		COBRA	
	met	unmet	uncut	cut
CCNA1	46	67		
TSCC22	87	190		
CLMN	185	96		
EML1	130	162		
HRASLS3			109	72 and 37
MCC			86	62 and 24
RARRES1			115	87 and 28
LOH11CR2A			129	80 and 49

Table 3: Frequency of methylation status of candidate genes promoter in culture NPC cells and control leukocytes

	NPC cells			Normal leukocytes		
	presence	absence	N	presence	absence	N
CCNA1	16	11	27	0	20	20
RARRES1	2	8	10	0	20	20
HRASLS3	2	8	10	0	20	20
EML1	0	10	10	0	10	10
CLMN	0	10	10	0	10	10
TSC22	0	10	10	0	10	10
MCC	0	10	10	0	10	10
LOH11CR2A	28	0	28	10	0	10

Table 4: Frequency of methylation status of candidate tumor suppressor genes in NPC comparing to control nasopharyngeal epithelium and leukocytes

Gene	Sample	Promoter hypermethylation		Total
		Absence	Presence	
CCNA1	Normal leukocytes	30	0	30
	Normal nasopharyngeal epithelium	20	0	20
	NPC biopsy	39	51	90
	Cultured NPC cells	0	9	9
RARRES1	Normal leukocytes	30	0	30
	Normal nasopharyngeal epithelium	20	0	20
	NPC biopsy	41	48	89
	Cultured NPC cells	1	5	6
HRASLS3	Normal leukocytes	30	0	30
	Normal nasopharyngeal epithelium	20	0	20
	NPC biopsy	73	23	96
	Cultured NPC cells	5	1	6

Table 5: Analysis of clinicopathological correlation to the promoter hypermethylation of the putative tumor suppressor genes in NPC

	Methylation status								
	RARRES1			*HRASLS3*			*CCNA1*		
	+	-	P	+	-	P	+	-	P
Tumor stage									
I / II	11	13	0.65	5	19	1.00	12	12	0.9
III / IV	12	9		3	14		10	12	
Nodal stage									
0	6	4	0.39	1	6	0.6	4	5	0.6
I/II/III	17	18		7	27		18	20	
Overall stage									
I / II	3	2	1.00	0	5	0.56	1	3	0.67
III / IV	20	20		8	28		21	21	
WHO									
II	9	10	0.97	5	12	0.45	8	11	0.72
III	11	15		4	20		14	13	

CHAPTER V
DISCUSSION

Currently, comprehensive gene expression profiling of individual cancer type has become one of the most popular ways to analyze individual cancer. We here apply the information derived from the gene expression analysis and to expand this knowledge to identify potential tumor suppressor genes in NPC. Integrating one known feature of tumor suppressor genes, namely LOH to the genome-wide expression analysis, allowed us to identify 3 potential tumor suppressor genes in NPC, *CCNA1*, *HRASLS3*, and *RARRES1*. These genes have been proven to be relatively under-expressed and preferentially methylated at the promoter in NPC comparing to normal tissue. Furthermore, these three genes have been shown previously to be putative tumor suppressor genes in other cancer type, for example *HRASLS3* in lung cancer, *RARRES1* in lung cancer, prostrate cancer, and head and neck cancer.(16) The above findings not only corroborate the results from our previous microarray study but also helps in elucidating the molecular epigenetic changes underlying the NPC.

Firstly, we detected promoter methylation of 8 down-regulated genes in NPC biopsy and WBC. We found that only 3 genes; *CCNA1*, *HRASLS3* and *RARRES1* had promoter methylation in NPC but not in WBC. We could not detect promoter methylation of 4 genes, *EML1*, *TSC22*, *CLMN* and *MCC* in both NPC biopsy and WBC. The explanation is may be suppression of these 4 genes is caused by the other mechanism such as mutation. Moreover, we found that *LOH11CR2A* had promoter methylation in both NPC biopsy and WBC. Methylation of *LOH11CR2A* in both cells may be 1.) imprinting or 2.) tissue specific. We further detected promoter methylation of *CCNA1*, *HRASLS3* and *RARRES1* in NPC biopsy, NPC cell culture and normal tissue to test tumor specificity of these 3 genes. We could detect promoter methylation around 50% of *CCNA1* and *RARRES1*, 20% of *HRASLS3* in NPC biopsy and 100% of *CCNA1*, 83% of *RARRES1* and *17%* of *HRASLS3*. This result indicated that promoter methylation of *CCNA1* and *RARRES1* could be the marker for NPC tumorigenesis, especially *RARRES1*; because there was the other group also reported 90% promoter methylation of *RARRES1* in NPC. Promoter methylation is the one mechanism which can inactivate

CCNA1 and *RARRES1* but not only methylation but also mutation can inactivate these 2 genes. We could detect only 17% methylation of *HRASLS3* in NPC biopsy. That may be *HRASLS3* is not the important gene in NPC tumorigenesis.

Function of Cyclin A1 is unclear, whether it behaves as an oncogene or a tumor suppressor gene is currently unknown. However, Cyclin A1 is believed to play a role in oncogenesis because of its involvement in cell cycle progression. Moreover, high levels of Cyclin A1 have been found in acute leukemia cell lines and myeloid leukemia samples. (81,90) More recently, it has been implicated that cyclin A1 might be involved in DNA double strand break repair by interacting with Ku70.(97) (In addition, there are several recent reports documenting this gene to be down regulated and hypermethylated in squamous cell carcinomas, such as those of the head and neck and the cervical locations.(110, 111) Here, we retrieved the data from global gene expression profile of NPC to search for candidate tumor suppressor gene and of interest *CCNA1* was indicated to be down-regulated. Furthermore, we found that its promoter region was methylated in a sub-set of NPC tissues but not in normal nasopharyngeal swab and leukocyte. Because transcriptional silencing by promoter methylation is a common mechanism for inactivation of tumor suppressor gene, taken together, Cyclin A1 may play a role as a tumor suppressor gene in squamous cell cancers including NPC.

HRASLS3 (H-ras like suppressor 3) is a class II tumor suppressor gene which is down-regulated in various cancer cell lines derived from melanoma, neuroblastoma, adenocarcinoma, and fibrosarcoma. (103) There is evidence that promoter methylation of this gene in lymphoma cells can result in gene silencing. (107) From our microarray data, *HRASLS3* is the gene, which is down regulated in NPC and in this study, we detected promoter methylation of this gene in NPC, which might be responsible for the inactivate of this putative tumor suppressor gene in NPC.

RARRES1 (retinoic acid receptor responsive element) is a candidate tumor suppressor gene of human prostate cancer, head and neck cancer and lung cancer, and recent evidence suggesting that the promoter of this gene is methylated in several of these cancers such as esophageal, endometrial, head and neck and lung cancer. (98) In deed, promoter methylation of this gene has been detected in head and neck squamous

cell carcinoma but not in normal tissue. Recently, other groups studying this gene detected 90.7 % promoter methylation in primary NPC (99), here we also detected 51% promoter methylation in primary tumor and 83% in NPC cells. In addition, our microarray data revealed that this gene was down regulated in NPC; therefore *RARRES1* may be the important tumor suppressor gene in this cancer type.

In conclusion, we report three putative tumor suppressor genes, *CCNA1*, *RARRES1* and *HRASLS3*, in NPC identified from the analysis of available gene expression profiling and alleotyping database and, the status of promoter hypermethylation. These 3 genes might be molecular markers for NPC because their promoters are methylated in NPC and not in normal cells. Further expanding the analysis in broader panel of genes from the database and functional study is warranted in the future.

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APPENDIX

APPENDIX

BUFFERS AND REAGENT

1. Lysis Buffer 1

Sucrose	109.54	g
1.0 M Tris – HCl (pH 7.5)	10	ml
1.0 M $MgCl_2$	5	ml
Triton X – 100 (pure)	10	ml
Distilled water to	1,000	ml

Sterilize the solution by autoclaving and store in a refrigerator (at 4^0C).

2. Lysis Buffer 2

5.0 M NaCl	15	ml
0.5 M EDTA (pH 8.0)	48	ml
Distilled water to	1,000	ml

Sterilize the solution by autoclaving and store at room temperature.

3. 10% SDS solution

Sodium dodecyl sulfate	10	g
Distilled water to	100	ml

Mix the solution and store at room temperature.

4. 20 mg/ml Proteinase K

Proteinase K	2	mg
Distilled water to	1	ml

Mix the solution and store in a refrigerator (at -20^{0}C).

5. 1.0 M Tris – HCl

Tris base	12.11	g

Dissolve in distilled water and adjusted pH to 7.5 with HCl

Distilled water to	100	ml

Sterilize the solution by autoclaving and store at room temperature.

6. 0.5 M EDTA (pH 8.0)

Disodium ethylenediamine tetraacetate.2H_2O	186.6	g

Dissolve in distilled water and adjusted pH to 8.0 with NaOH

Distilled water to	1,000	ml

Sterilize the solution by autoclaving and store at room temperature.

7. 1.0 M $MgCl_2$ solution

Magnesium chloride.6H_2O	20.33	g
Distilled water to	100	ml

Dispense the solution into aliquots and sterilize by autoclaving.

8. 5 M NaCl solution

Sodium chloride	29.25	g
Distilled water to	100	ml

Dispense the solution into aliquot and sterilize by autoclaving.

9. 10X Tris borate buffer (10X TBE buffer)

Tris – base	100	g
Boric acid	55	
g0.5 M EDTA (pH 8.0)	40	
ml		

Adjust volume to 1,000 ml with distilled water. The solution was mixed and store at room temperature.

10. 6X loading dye

Bromphenol blue	0.25	g
Xylene cyanol	0.25	g
Glycerol	50	ml
1M Tris (pH 8.0)	1	ml
Distilled water until	100	ml

Mixed and stored at 4^{0}C

11. 7.5 M Ammonium acetate (CH_3COONH_4)

Ammonium acetate	57.81	g
Distilled water	80	ml

Adjust volume to 100 ml with distilled water and sterilize by autoclaving.

12. 25:24:1 (v/v) Phenol-chloroform-isoamyl alcohol

Phenol	25	volume
Chloroform	24	volume
Isoamyl alcohol	1	volume

Mix the reagent and store in a sterile bottle kept in a refrigerator.

13. 12% Non-denature acrylamide gel (w/v)

40%acrylamide: Bis (19:1)	3	ml
5X TBE	1	ml
10% ammoniumpersulfate	105	µl
TEMED	8	µl
H_2O	6	ml

Dissolve by heating in microwave oven and occasional mix.

14. TE buffer

Tris base	1.21	g
5M EDTA	200	µl

Adjust pH to 7.5 with conc.HCL and adjust volume to 1.0 litre with H_2O.

15. 3 M Sodium acetate (CH_3COONH_4)

Sodium acetate	40.82	g
dH2O	80	ml

adjust the pH to 5.3 by adding conc. HCl

Adjust volume to 100 ml with dH_2O, and sterile by autoclaving

16. LB broth

Tryptone	10	g
Yeast extract	5	g

NaCl	10	g

Adjust the pH to 7.0 by adding 5N NaOH

Adjust volume to 1.0 litre with dH_2O with sterile water, and sterilize by autoclaving

17. LB agar

Tryptone	10	g
Yeast extract	5	g
NaCl	*10*	*g*
Agar	*10*	g

Adjust the pH to 7.0 by adding 5N NaOH

Adjust volume to 1.0 litre with dH_2O with sterile water, and sterilize by autoclaving

18. Sodium Bisulfite

Sodium Bisulfite	3.76	g
dH_2O	10	ml

adjust the pH 5.0 by adding 10 M NaOH

19. Hydroquinone

Hydroquinone	55.4	mg
dH_2O	50	ml

Protected from light by cover with foil

BIOGRAPHY

Miss Pattamawadee Yanatatsaneejit was born on October 31, 1972 in Bangkok. She received her Master Degree of Science (Medical Science) from Chulalongkorn University in 2002. She has a position as lecturer at Faculty of Science, Chulalongkorn University.